

Maintenance of murine platelet homeostasis by the kinase Csk and the phosphatase CD148

Jun Mori,^{1*} Zoltan Nagy,^{1*} Giada Di Nunzio,¹ Christopher W. Smith,¹ Mitchell J. Geer,¹
Rashid Al Ghaithi,² Johanna P. van Geffen,³ Silke Heising,¹ Luke Boothman,¹ Bibian M. E.
Tullemans,³ Joao N. Correia,¹ Louise Tee,¹ Marijke J. E. Kuijpers,³ Paul Harrison,² Johan W.
M. Heemskerk,³ Gavin E. Jarvis,⁴ Alexander Tarakhovsky,⁵ Arthur Weiss,⁶ Alexandra
Mazharian¹ and Yotis A. Senis¹

¹Institute of Cardiovascular Sciences and ²Institute of Inflammation and Ageing, College of Medical and Dental Sciences, University of Birmingham, Birmingham, UK; ³Department of Biochemistry, Cardiovascular Research Institute Maastricht, Maastricht University, Maastricht, The Netherlands; ⁴Department of Physiology, Development and Neuroscience, University of Cambridge, Cambridge, UK; ⁵Laboratory of Immune Cell Epigenetics and Signaling, The Rockefeller University, New York, USA; ⁶Department of Medicine, Rosalind Russell-Ephraim P. Engleman Rheumatology Research Center and Howard Hughes Medical Institute, University of California, San Francisco, USA

*JM and ZN contributed equally to this study

Correspondence: Yotis A. Senis, Institute of Cardiovascular Sciences, College of Medical and Dental Sciences, University of Birmingham, Birmingham, B15 2TT, UK; e-mail: y.senis@bham.ac.uk

Short title: Csk and CD148 regulate platelet homeostasis

Word count Abstract: 171 words

Word count of main text: 4,932

Total number of figures and tables: 1 Table and 7 Figures (1 Supplemental Table, 10 Supplemental Figures and 3 Videos)

Key Points

- Csk and CD148 are non-redundant regulators of SFKs in platelets and deletion of either induces cell-intrinsic negative feedback mechanisms
- Csk is a negative regulator of SFK activity, whereas CD148 is a dual positive and negative regulator of SFK activity in platelets

Abstract

Src family kinases (SFKs) coordinate the initiating and propagating activation signals in platelets, however it remains unclear how they are regulated. Here we show that ablation of C-terminal Src kinase (Csk) and receptor-like protein tyrosine-phosphatase CD148 in mice results in a dramatic increase in platelet SFK activity, demonstrating that these proteins are essential regulators of platelet reactivity. Paradoxically, Csk/CD148-deficient mice exhibit reduced *in vivo* and *ex vivo* thrombus formation and increased bleeding following injury, rather than a prothrombotic phenotype. This is a consequence of multiple negative feedback mechanisms, including downregulation of the immunoreceptor tyrosine-based activation motif (ITAM)- and hemi-ITAM-containing receptors GPVI-FcR γ -chain and CLEC-2, respectively and upregulation of the immunoreceptor tyrosine-based inhibition motif (ITIM)-containing receptor G6b-B and its interaction with the tyrosine phosphatases Shp1 and Shp2. Results from an analogue-sensitive Csk mouse model demonstrate the unconventional role of SFKs in activating ITIM signaling. This study establishes Csk and CD148 as critical molecular switches controlling the thrombotic and hemostatic capacity of platelets, and reveals cell-intrinsic mechanisms that prevent pathological thrombosis from occurring.

Introduction

Platelets are highly reactive fragments of megakaryocytes (MKs) that interrogate the vessel wall and prevent excessive blood loss following injury. They do so by adhering to exposed extracellular matrix proteins and forming a thrombus that transiently occludes the blood vessel, promoting wound repair and vessel regeneration. However, it remains unclear how the reactivity of platelets in the circulation is regulated.

Platelets contain high levels of Src family kinases (SFKs) that have a coordinating role in initiating and propagating primary activation signals.^{1,2} The three most abundant SFKs in human and mouse platelets are Src, Lyn and Fyn (Figure S1).^{3,4} Src and Fyn act primarily as positive regulators of platelet activation,⁵⁻⁸ whereas Lyn is both a positive and negative regulator of activation.^{9,10} SFKs are constitutively associated with the cytoplasmic tails of several important platelet receptors, including: the glycoprotein (GP)Ib-IX-V complex,^{11,12} the immunoreceptor tyrosine-based activation motif (ITAM)-containing GPVI-FcR γ -chain receptor complex,^{5,13} and the integrin α IIb β 3.^{14,15} SFKs are also involved in transmitting secondary activation signals from G protein-coupled receptors.¹⁶⁻²¹

Equally important, but less well understood are the inhibitory functions of SFKs. Concomitant with phosphorylating ITAM-containing receptors activating platelets,^{5,22} SFKs also phosphorylate immunoreceptor tyrosine-based inhibition motif (ITIM)-containing receptors that inhibit platelets.²³ The latter recruit SH2 domain-containing phosphatases, including the non-transmembrane protein-tyrosine phosphatases Shp1 and Shp2,²⁴⁻²⁶ that inhibit platelet activation. The two most well characterized platelet ITIM-containing receptors are PECAM-1 and G6b-B, which inhibit platelet activation and modulate platelet homeostasis, respectively.^{27,28}

Limited knowledge has been gained how platelet SFKs are regulated. SFKs are primed by dephosphorylation of the C-terminal inhibitory phospho-tyrosine residue by the

receptor-like tyrosine phosphatase CD45 in most hematopoietic cells,²⁹ and by CD148 in platelets.³⁰⁻³² Primed SFKs need to be fully activated through *trans*-auto-phosphorylation of a tyrosine residue located within the catalytic domain.³³ On the other hand, inactivation of SFKs can occur through the action of C-terminal Src kinase (Csk) and the structurally-related Csk homologous kinase (Chk), also referred to as megakaryocyte-associated tyrosine kinase (Matk), both of which target the C-terminal inhibitory tyrosine residue in SFKs.^{34,35} However, it remains unknown how the threshold of individual SFK activation is set in platelets

To address these questions, we generated a series of MK-specific conditional *knockout* (KO) mouse models leading to deletion of *Csk*, *CD148* or *Csk/CD148*, and studied the consequences of these deletions for platelet physiology. Disruption of the homeostatic balance of SFK activity in MKs had dramatic and unexpected consequences on the number and reactivity of platelets in the circulation, due to cell-intrinsic negative feedback mechanisms, involving the ITIM-containing receptor G6b-B and Chk, culminating in anti-thrombotic and hemorrhagic outcomes.

Materials and methods

Mice

Csk^{fl/fl}, *CD148^{fl/fl}*, *Pf4-Cre⁺* and *Csk^{AS}* mice were generated, as previously described.³⁶⁻³⁹ All mice were on a C57BL/6 background. All procedures were undertaken with United Kingdom Home Office approval in accordance with the Animals (Scientific Procedures) Act of 1986.

Antibodies and reagents

Thiazole orange (BD Retic-Count™) were from BD Bioscience. PP1 analogue IV (3-IB-PP1) was from Calbiochem. Antibodies are described in detail in Supplemental Methods.

Immune thrombocytopenia

Thrombocytopenia was induced as previously described.²⁸

Platelet preparation

Blood was collected from terminally CO₂-narcosed mice from the abdominal vena cava into 1:10 (v:v) acid-citrate-dextrose anticoagulant. Washed platelets were prepared as previously described.⁴⁰ Adenosine diphosphate (ADP)-sensitive washed platelets were prepared in the presence of ADP scavenger apyrase as previously described.⁴¹ Platelet counts were normalized and used for spreading (2×10^7 /ml), aggregation (2×10^8 /ml) or biochemical analysis ($4 - 5 \times 10^8$ /ml).

Platelet functional assays and biochemistry

Platelet aggregation, adenosine triphosphate (ATP) secretion, spreading, clot retraction, platelet adhesion under flow, total thrombus-formation analysis system (T-TAS), stimulation

for biochemical analysis, immunoblotting and immunoprecipitation are described in detail in Supplemental Methods.

Flow cytometry

Reticulated platelets in blood were double stained with α IIb and thiazole orange (BD Retic-CountTM, BD Bioscience). Surface protein expression was measured in blood or bone marrow cells with indicated FITC- or PE-conjugated antibodies by flow cytometry (BD Accuri C6 for platelets; BD FACSCalibur for bone marrow cells), as previously described.^{25,28}

Tail bleeding assay

Experiments were performed on 8-10 week old *KO* and litter-matched *WT* mice as previously described.³⁰

***In vivo* thrombosis assays**

Laser-induced injury of arterioles in the cremaster muscle and ferric chloride-induced injury of carotid arteries were performed and analyzed as previously described.⁴²

Statistical analysis

Data presented are means \pm standard errors of the mean (SEM). One-way or two-way ANOVA followed by *post-hoc* tests were used to determine statistical significance ($P < .05$). Further details on statistical analysis are provided in Supplemental Methods.

Results

Rescue of thrombocytopenia in *Csk*/*CD148*-deficient mice

MK-specific *Csk* and *CD148* conditional *KO* (*Csk* and *CD148 KO*) mice were generated by crossing *Csk^{fl/fl}* and *CD148^{fl/fl}* mice with *Pf4-Cre⁺* transgenic mice, respectively.³⁶⁻³⁸ *Csk/CD148* conditional *double KO* (*DKO*) mice were also generated, to determine if deletion of both enzymes simultaneously would rescue phenotypes of single *KO* mice, and to reveal novel mechanisms of SFK regulation (Figure 1A).

Platelet count in *Csk KO* mice was reduced by 65% and platelet volume increased by 33% (Figure 1B and Table S1), suggesting defects in platelet production and/or clearance. This was supported by a 2-fold increase in the proportion of reticulated, immature platelets in these mice (Figures 1C and S2A). The same parameters were normal in *CD148 KO* mice (Figures 1B-C and S2A). While the platelet count was normal in *DKO* mice, the variability in numbers was greater than in *wild-type* (*WT*) and *Csk KO* mice, and the proportion of large, reticulated platelets was similar to that in *Csk KO* mice (Figures 1B-C, S2A and Table S1). Increased numbers of immune and granulocytic cells in *Csk* and *DKO* mice (Table S1) may be an indirect consequence of non-specific deletion of floxed *Csk* and *CD148* in other hematopoietic lineages by the *Pf4-Cre* transgene, which is reported to be 'leaky'.⁴³ Interestingly, the proportion of P-selectin⁺αIIb⁺ platelets were normal in *Csk KO* mice and only marginally elevated in *DKO* mice (Figure S2B), pointing to only minimal pre-activation of these platelets in the circulation or downregulation of activation markers.

To explore whether increased platelet size and proportion of young platelets correlate with changes in other platelet parameters, we quantified levels of receptors that regulate platelet production and activation. Notably several receptors we focused on, including αIIbβ3, GPIbα, GPVI-FcR γ-chain, CLEC-2 and G6b-B rely on SFKs to transmit signals.² Surface levels of integrin αIIb were marginally increased in *DKO* platelets, whereas GPIbα

levels were increased by 63% and 38% in *Csk KO* and *DKO* platelets, respectively (Figure 1Di). Surface levels of GPVI were reduced by 77%, 44% and 88% in *Csk KO*, *CD148 KO* and *DKO* platelets, respectively, correlating with total protein levels of GPVI and FcR γ -chain (Figure 1Di-ii). Surface and total protein levels of CLEC-2 were reduced in *Csk KO* and *DKO* platelets, which were significantly more pronounced in *DKO* (Figure 1Di-ii). In contrast, surface expression of the inhibitory receptor G6b-B was increased by 61% and 73% in *Csk KO* and *DKO* platelets, respectively, correlating with total protein levels (Figure 1Di-ii). Surface levels of the integrin $\alpha 2$ subunit and metalloproteinase ADAM10 that mediates shedding of GPVI were normal (Figure S3A). Intriguingly, expression of surface receptors in αIb^+ bone marrow cells was normal in all three genotypes (Figure S3B), suggesting auto-regulation of these receptors in platelets. Collectively, these findings demonstrate downregulation of the (hemi-)ITAM-containing activation receptors GPVI-FcR γ -chain and CLEC-2, and concomitant upregulation of the ITIM-containing inhibitory receptor G6b-B in *Csk*-deficient platelets.

To investigate the cause of low platelet counts, we measured the rate of platelet recovery following anti-GPIb α antibody-mediated platelet depletion. This was significantly reduced in *Csk KO* mice, marginally reduced in *CD148 KO* and normal in *DKO* mice (Figure 1Ei-ii). The clearance of biotinylated platelets was normal in mice of all three genotypes (Figure S4A-B). Both *Csk KO* and *DKO* mice exhibited splenomegaly (Figure S5A-B). Elevated MK counts were observed in spleens in all three genotypes, which was associated with myelofibrosis (Figure S6A). MK counts were moderately elevated in the bone marrow of *Csk KO* mice, but with no associated myelofibrosis (Figure S6B). We observed more P-selectin $^+$ αIb^+ hematopoietic cells in *Csk KO* and *DKO* mice (Figure S6C), which may

explain the presence of myelofibrosis in the spleen of these mice.⁴⁴ Together, these findings suggest defective MK activation in *Csk KO* and *DKO* mice.

Hemostatic and thrombotic defects in *Csk*/CD148-deficient platelets

We hypothesized that *Csk KO* mice would be predisposed to thrombosis, due to increased SFK activity, rendering platelets hyperactive. To test this, we analyzed mice of all three genotypes in established *in vivo* models of hemostasis and thrombosis. Paradoxically, *Csk KO* mice exhibited increased bleeding in a tail injury model (Figure 2A). *CD148 KO* mice exhibited normal hemostasis, whereas *DKO* mice had increased bleeding compared to *Csk KO* mice (Figure 2A). Increased bleeding in *Csk KO* and *DKO* mice could not be explained solely by reduced platelet count, which was normal in *DKO* mice.

To explore the kinetics of arterial thrombus formation, we employed the laser injury-induced thrombosis model in small arterioles in mice. Thrombus formation and fibrin deposition were moderately and severely reduced in *Csk KO* and *DKO* mice, respectively (Figure 2Bi-iv and Videos 1-2). Thrombus formation was also severely compromised in *CD148 KO* mice, whereas fibrin deposition was only moderately reduced (Figure 2Bi-iv). Similar results were observed following FeCl₃-induced injury of the carotid artery, albeit thrombus formation was marginally better in *CD148 KO* compared with *Csk KO* mice (Figure 2Ci-iii and Video 3). These results demonstrate that the ability of *Csk KO* and *DKO* mice to form thrombi *in vivo* is markedly reduced, resulting in increased bleeding.

Aberrant platelet function underlies thrombotic and defects

To determine the cause of reduced thrombus formation observed in the different *in vivo* models, we employed the T-TAS microfluidic device, which can differentiate between platelet- and coagulation-driven defects. We found that platelets of all three genotypes

displayed severe defects in forming stable platelet thrombi on collagen surface, as we were unable to detect an increase in the flow pressure (Figure 3Ai). However, under conditions where platelets were flowed over collagen in the presence of tissue factor to maximize coagulation, platelets of all three genotypes formed equally stable thrombi (Figure 3Aii), suggesting coagulation is unaltered in these mice and that defects in thrombus formation are intrinsic to platelets.

To identify specific platelet defects, we measured multiple parameters of platelet function and thrombus formation *ex vivo* at arterial shear rates on collagen I, von Willebrand factor-binding peptide (vWF-BP) and laminin, and vWF-BP, laminin and the snake venom rhodocytin, which induces CLEC-2 signaling. We found markedly decreased platelet deposition and thrombus formation on collagen I in *Csk KO* and *CD148 KO* samples, which were further reduced in *DKO* samples (Figure 3Bi-iii and vi). α -granule secretion, integrin α Ib β 3 activation and phosphatidylserine (PS) exposure (determined as P-selectin, JON/A and Annexin V staining, respectively) followed the same trends (Figure 3Biv-vi). This likely is a consequence of markedly reduced surface levels of GPVI-FcR γ -chain, defective SFK signaling and increased inhibitory mechanisms. We found no difference between genotypes in platelet deposition on the vWF-BP and laminin surface on which adhesion is mediated by platelet GPIb α via immobilized plasma vWF and platelet integrin α 6 β 1, which binds laminin,⁴⁵ suggesting *Csk* and *CD148* do not play major roles regulating GPIb α or α 6 β 1 signaling pathways (Figure 3Bi-ii and vi). Upon complementing the vWF-BP and laminin surface with the CLEC-2 ligand rhodocytin, which drives a more robust platelet deposition,⁴⁶ adhesion was significantly reduced in the three *KOs* (Figure 3Bi-ii, vi). Notably, CLEC-2 expression was marginally and markedly reduced in *Csk KO* and *DKO* platelets, respectively,

demonstrating that Csk and CD148 participate in SFK regulation during CLEC-2-evoked hemi-ITAM signaling, as previously described.³²

We next analyzed platelets of all three genotypes in a range of *in vitro* functional assays. Consistent with previous findings, *CD148 KO* platelets exhibited reduced aggregation and ATP secretion to collagen and CRP stimulation, which was overcome at high concentrations of CRP (Figure 3Ci-ii). *Csk KO* and *DKO* platelets also exhibited reduced reactivity to collagen, and failed to respond to collagen-related peptide (CRP) (Figure 3Ci-ii). We also investigated the aggregation and ATP secretion response of platelets to anti-CLEC-2 antibody-mediated activation. *CD148 KO* platelets exhibited an increase in the lag time at low concentration of anti-CLEC-2 antibody, and responded normally to a high concentration (Figure 3Ciii). In contrast, *Csk KO* and *DKO* platelets exhibited shortened lag times in response to anti-CLEC-2 antibody, despite expressing lower levels of CLEC-2 receptor (Figures 1Di-ii and 3Ciii). Platelets from all three mouse models responded normally to thrombin and TxA₂ analogue U46619 (Figure 3Civ-v). As expected, *WT* platelets prepared in the absence of apyrase did not respond to 10 μ M ADP, due to P2Y₁ and P2Y₁₂ receptor desensitization (Figure 3Cvi); however, *Csk KO* and *DKO* platelets were able to aggregate and secrete ATP in response to the same concentration of ADP (Figure 4Cvi), suggesting reduced P2Y₁ and P2Y₁₂ internalization or increased signaling via these receptors, both of which utilize SFKs to transmit signals.² Indeed, washed platelets prepared in the presence of apyrase, preventing receptor desensitization, responded normally to 3 and 10 μ M ADP, except for *DKO* platelets, which hyper-responded (Figures 3Cvii and S7), supporting the hypothesis of increased P2Y₁₂ signaling.

We investigated platelet adhesion and spreading on fibrinogen, which is dependent on SFK-mediated α Ib β 3 outside-in signaling and cytoskeletal remodeling. As expected, *Csk*-deficient platelets spread to a greater extent than *WT* platelets, whereas CD148-deficient

platelets exhibited reduced spreading (Figure 3Di-ii). However, *DKO* platelets spread comparably to *WT* platelets, suggesting that negative feedback mechanisms were activated in *DKO* platelets. When platelets were pre-activated with thrombin, spreading of *Csk KO* and *DKO* platelets were significantly increased compared to *WT* (Figure 3Di-ii).

To further test the role of Csk and CD148 in $\alpha\text{IIb}\beta 3$ signaling, we assessed clot retraction in these mice *in vitro*, revealing a significant reduction in clot retraction of *CD148 KO* platelets (Figure 3E). These results strongly suggest an important role for functional Src kinase activity in clot retraction.

Increased SFK activity in Csk/CD148-deficient platelets

To determine the mechanism underlying the hypo-thrombotic phenotypes, we assessed SFK activity in unstimulated platelets of all three genotypes. We measured *trans*-auto-phosphorylation of the activation loop tyrosine residue of SFKs (Src p-Tyr418) as an indirect indicator of SFK activity in resting platelets by quantitative capillary electrophoresis-based immunoassays (ProteinSimple Wes). As expected, deletion of Csk resulted in a significant increase in Src p-Tyr418, whereas deletion of CD148 resulted in a marked decrease in Src p-Tyr418 (Figure 4Ai-ii). Deletion of both Csk and CD148 resulted in an unexpected overall increase in Src p-Tyr418, demonstrating high activity SFKs in these platelets (Figure 4Ai-ii and B).

Phosphorylation of the C-terminal inhibitory tyrosine residues of Lyn (Tyr507), Src (Tyr529) and Fyn (Tyr530) is typically inversely related to SFK activity and to Src p-Tyr418. Indeed, phosphorylated Lyn Tyr507 (Lyn p-Tyr507) was not detected in resting Csk-deficient platelets (Figure 4Ai-ii), and phosphorylation of Src Tyr529 (Src p-Tyr529) and Fyn Tyr530 (Fyn p-Tyr530) were markedly decreased, suggesting that Csk is the main kinase that phosphorylates these residues and attenuates the activity of these SFKs. Conversely,

phosphorylation of all three inhibitory tyrosine residues was increased in resting CD148-deficient platelets (Figure 4Ai-ii), confirming previous findings that CD148-induced dephosphorylation activates SFKs in platelets.³¹ A partial rescue of Lyn p-Tyr507, Src p-Tyr529 and Fyn p-Tyr530 was observed in *DKO* platelets (Figure 4Ai-ii), suggesting that in the absence of Csk and CD148 another kinase phosphorylates these residues, the obvious candidate being Chk, which was upregulated in *Csk KO* and *DKO* platelets (Figure 4C), explaining the rescue of inhibitory site phosphorylation of SFKs in *DKO* platelets. Expression of the tyrosine phosphatases PTP-1B, Shp1 and Shp2, all of which have been implicated in regulating SFK activity were normal in all three genotypes (Figure 4C). Interestingly, SFK activity (Src p-Tyr418) was highest in *DKO* platelets, despite increased phosphorylation of the C-terminal inhibitory tyrosine residues of Lyn, Src and Fyn, compared with *Csk KO* platelets. It remains to be determined whether phosphorylation of the activation loop and C-terminal inhibitory tyrosine residues co-exist in the same molecules or in distinct pools of SFKs. Dually phosphorylated SFKs have been reported to be active in T cells.⁴⁷

We next assessed whether SFK activity in MKs is comparable to platelets. Indeed, SFKs were similarly phosphorylated in starved BM-derived MKs (Figure S8A-B) as they were in resting platelets of the same genotype (Figure 4Ai-ii), compatible with increased SFK activity in *Csk KO* and *DKO* MKs, and decreased SFK activity in *CD148 KO* MKs, which may underlie aberrant MK function and myelofibrosis in *Csk KO* and *DKO* mice.

Collectively, these findings demonstrate that Csk is an inhibitor of SFK activity in platelets and MKs. The finding that *DKO* platelets display markedly elevated SFK activity compared to *Csk KO* platelets suggest that CD148 can act as a dual activator and inhibitor of SFK activity in platelets and MKs (Figure 4B).

ITAM and integrin receptor signaling is differentially regulated by Csk and CD148

To determine why platelets with high SFK activity were hypo-reactive, we investigated tyrosine phosphorylation downstream of GPVI-FcR γ -chain, CLEC-2 and α IIb β 3, all of which rely on SFKs and Syk to initiate and propagate signaling. Due to the dramatic reduction of GPVI-FcR γ -chain expression in *Csk KO* and *DKO* platelets (Figure 1Di-ii), platelets were stimulated with a high concentration of collagen (30 μ g/ml), which signals primarily through GPVI-FcR γ -chain. Basal whole cell tyrosine phosphorylation (p-Tyr) and Src p-Tyr418 were significantly increased in resting *DKO* platelets and collagen-stimulated *Csk KO* and *DKO* platelets compared with *WT* platelets (Figure 5Ai-iii and S9A). In contrast, basal and collagen-mediated Src p-Tyr418 was significantly reduced in *CD148 KO* platelets compared with *WT* platelets (Figure 5Ai-iii and S9A). Induced phosphorylation of the FcR γ -chain, which is directly mediated by SFKs and acts as a docking site for Syk, was less pronounced in *Csk KO* and *DKO* platelets (Figure 5Ai) due to reduced expression of the FcR γ -chain in these platelets (Figure 1Dii). Syk activation was indirectly measured as phosphorylation of Syk tyrosine residues 525 and 526 (Syk p-Tyr525/6), and correlated directly with FcR γ -chain levels and phosphorylation. Syk p-Tyr525/6 was highest in collagen-stimulated *WT* platelets, and increased only marginally in collagen-stimulated platelets of all three genotypes (Figure 5Aii-iii and S9A), despite *Csk KO* and *DKO* platelets having high SFK activity. These findings support a model in which receptor-mediated membrane localization of Syk is essential for activation.

Platelets were also stimulated with a high concentration of anti-CLEC-2 antibody (10 μ g/ml), mimicking podoplanin-mediated cross-linking of the receptor. The pattern and intensity of whole cell p-Tyr and SFK phosphorylation generally mirrored that of collagen-stimulation in the various genotypes (Figure 5Bi-iii and S9B). However, Syk p-Tyr525/6

was much higher in *Csk KO* platelets compared with any of the other genotypes (Figure 5Bii-iii and S9B), despite reduced CLEC-2 expression. Despite the reduced CLEC-2 and GPVI-FcR γ -chain levels in *DKO* platelets, stimulation by anti-CLEC-2 antibody resulted in normal Syk p-Tyr525/6, whereas stimulation by collagen led to significantly reduced Syk phosphorylation, correlating with normal and reduced aggregation, respectively.

We also investigated α IIB β 3 signaling to determine the cause of increased spreading of *Csk KO* platelets on fibrinogen (Figure 3Di-ii). To initiate signaling, α IIB β 3 relies mainly on Src, and to a lesser extent Fyn and is localized exclusively in non-lipid rafts.^{8,15,48,49} Whole cell p-Tyr was increased in fibrinogen-adhered *Csk KO* and *DKO* platelets (Figure 5Ci), demonstrating a general increase in outside-in integrin signaling in these platelets. In agreement with increased spreading of *Csk KO* platelets, but normal spreading of *DKO* platelets on fibrinogen (Figure 3Di-ii, basal), we found increased SFK and Syk activities in *Csk KO* platelets, but not significantly higher SFK and Syk activities in *DKO* platelets (Figure 5Cii-iv and S9C).

In addition to the elevated expression of the ITIM-containing receptor G6b-B in *Csk KO* and *DKO* platelets (Figure 1Di-ii), we found a marked increase in G6b-B tyrosine phosphorylation and binding of the tyrosine phosphatases Shp1 and Shp2 under resting and collagen-stimulated conditions (Figure 5Di), suggesting increased inhibitory signaling via the G6b-B-Shp1-Shp2 complex in *Csk KO* and *DKO* platelets (Figure 5Dii). Thus, increased SFK activity in *Csk KO* and *DKO* platelets leads to compensatory upregulation of inhibitory ITIM signaling and parallel downregulation of the (hemi-)ITAM-containing GPVI-FcR γ -chain and CLEC-2 receptors, explaining the reduced activity of these platelets.

Csk is a critical inhibitor of ITAM- and integrin-mediated platelet activation

To circumvent developmental and compensatory mechanisms arising from deletion of Csk, we utilized a transgenic mouse model expressing a PP1-analogue (3-IB-PP1) sensitive form of Csk (*Csk^{AS}*), enabling rapid and specific inhibition of *Csk^{AS}* in these mice.³⁹ Platelet count, volume and receptor expression were normal in *Csk^{AS}* mice (*data not shown*). Platelets from *Csk^{AS}* mice aggregated and secreted normally to all agonists tested in the presence of vehicle alone (dimethyl sulfoxide, DMSO) (Figure 6Ai-iv), and marginally more robustly to a sub-threshold concentration of anti-CLEC-2 antibody (3 µg/ml) in the presence of 10 µM 3-IB-PP1 (Figure 6Aiii). Inhibitor-treated *Csk^{AS}* platelets also spread marginally better than control on fibrinogen (Figure 6Bi-ii). Collectively, these findings validate that Csk is an inhibitor of both ITAM and integrin receptor mediated platelet functions. SFK activity (Src p-Tyr418) was significantly increased in resting, CRP (30 µg/ml) and anti-CLEC-2 antibody (10 µg/ml) stimulated *Csk^{AS}* platelets treated with 10 µM 3-IB-PP1 (Figures 6Ci-iii, Di-iii and S10A-B). However, Lyn p-Tyr507, Src p-Tyr529 and Fyn p-Tyr530 were only minimally reduced in the presence of 3-IB-PP1 (Figure 6Cii-iii, Dii-iii and S10A-B), suggesting that tyrosine phosphatases do not automatically dephosphorylate these residues in the absence of Csk. Syk activity was significantly increased at later time points post CRP-stimulation (Figure 6Cii-iii and S10A). Concomitantly, G6b-B was hyper-phosphorylated and had more associated Shp1 and Shp2 in collagen-stimulated *Csk^{AS}* platelets treated with 3-IB-PP1 (Figure 6E). As *G6b-B KO* platelets display markedly elevated Syk activity,²⁸ we hypothesized that the increased inhibitory signaling provided by G6b-B impeded further enhancement of CRP- and CLEC-2 antibody-mediated aggregation and overall tyrosine phosphorylation in *Csk^{AS}* platelets. These findings suggest that SFKs activate inhibitory pathways in parallel with activation pathways, to prevent platelet hyper-activation.

Discussion

Here we describe a fundamental mechanism controlling SFK activity in the MK lineage, involving the kinase Csk, and the phosphatase CD148. We show that Csk is a major inhibitor of SFKs in platelets, whereas CD148 primarily activates SFKs, but also attenuates SFK activity under as yet undefined conditions (Figures 4B and 7A). These functions of Csk and CD148 correlate with what has previously been described in immune cells.^{31,50-55} We show that lack of Csk in the MK lineage in mice results in increased SFK activity in platelets, paradoxical bleeding and reduced thrombosis due to negative feedback mechanisms, including downregulation of (hemi-)ITAM-containing receptors, and concomitant upregulation of the inhibitory ITIM-containing receptor G6b-B rendering platelets less responsive to vascular injury (Figure 7B). In contrast, deletion of CD148 results in reduced SFK activity and thrombus formation as a consequence of reduced (hemi-)ITAM and integrin receptor signaling. Bleeding was however normal in *CD148 KO* mice due to residual SFK activity and intact positive feedback mechanisms. Intriguingly, deletion of both Csk and CD148 markedly increased SFK activity, with more pronounced bleeding and thrombotic defects than in *Csk KO* mice, due to enhanced negative feedback effects (Figure 7B). A summary of the phenotypes of *Csk KO*, *CD148 KO* and *DKO* mice is provided in Table 1. Thus, sustained high SFK activity in platelets does not culminate in a general increase in platelet reactivity, but overcompensation of negative feedback mechanisms that attenuate the platelet response to various thrombogenic substrates. Moreover, reduced platelet count in *Csk KO* mice was not due to increased platelet clearance, but rather a reduction in platelet production, agreeing with a lack of activation and phagocytosis markers on platelets in these mice. Although mean platelet count was rescued in *DKO* mice, the variability in count, platelet volume and the proportion of reticulated platelets in the circulation suggested a less robust system controlling platelet homeostasis in the absence of Csk and CD148.

Negative feedback mechanisms activated in the *Csk KO* and *DKO* mice, including downregulation of the (hemi-)ITAM-containing receptors GPVI-FcR γ -chain and CLEC-2, and upregulation of the ITIM-containing receptor G6b-B and tyrosine kinase Chk, likely represent cell-intrinsic adaptation of platelets to high SFK activity. These findings are in agreement with the reduction of the ITAM-containing T cell receptor in *Csk*-deficient T cells,⁵⁶ and increased phosphorylation of the ITIM-containing receptor Sirp α , the lipid phosphatase SHP1 and Shp1 following inhibition of *Csk*^{AS} in immune cells.⁵⁷ A plausible explanation for the downregulation of (hemi-)ITAM-containing receptors is that these receptors are direct substrates of CD148; similarly, the ITAM-containing T cell receptor subunit CD3 ζ -chain is suggested to be a substrate of the tyrosine phosphatase CD45.^{58,59} A clear difference was observed in the downregulation of GPVI-FcR γ -chain and CLEC-2, suggesting differential regulation of these receptors. Whereas the GPVI-FcR γ -chain was severely reduced in both *Csk KO* and *DKO* platelets, CLEC-2 was markedly downregulated only in *DKO* platelets. This may be explained by the different affinities of Syk to different phospho-(hemi-)ITAMs,^{60,61} and thus the ability of the SH2 domains of Syk to protect tyrosine residues within these motifs from dephosphorylation by CD148, as proposed for the related kinase ZAP-70.⁶²⁻⁶⁴ We hypothesize, that the higher affinity of the tandem SH2 domains of Syk for the dual phospho-tyrosine residues of the FcR γ -chain ITAM in *Csk KO* platelets prevents CD148-mediated dephosphorylation. Sustained phosphorylation would in turn lead to internalization and degradation of the GPVI-FcR γ -chain complex. In contrast, due to the lower binding affinity of individual SH2 domains of Syk for single phospho-tyrosine residues with the hemi-ITAM of CLEC-2, Syk is less capable of protecting CLEC-2 from dephosphorylation by CD148. As a consequence, less CLEC-2 is destined for degradation than FcR γ -chain in *Csk KO* platelets. This is not however the case in *DKO*

platelets where no CD148 is present to dephosphorylate CLEC-2, hence it is markedly downregulated. Further work is needed to validate this hypothesis.

Analysis of tyrosine phosphorylation downstream of GPVI, CLEC-2 and $\alpha\text{IIb}\beta 3$ revealed that activation of Syk is not only dependent on SFK activity, but also on the presence of appropriate docking sites at the plasma membrane, providing evidence that increased SFK activity alone is insufficient to initiate downstream signaling. A growing body of evidence has established G6b-B as a major inhibitor of (hemi-)ITAM-containing receptor signaling in platelets, acting primarily at the level of Syk.^{28,65} It is therefore likely that increased G6b-B expression, phosphorylation, and Shp1 and Shp2 association contributes to the attenuation of (hemi-)ITAM signaling in *Csk KO* and *DKO* platelets. Upregulation of Chk in the absence of Csk likely also contributes to the attenuation of SFK-mediated signaling in these platelets, albeit less efficiently than its structurally related counterpart Csk. Although, Chk was previously reported in platelets,⁶⁶ it was not detected in either human or mouse platelets by mass spectrometry,^{3,4} in agreement with our findings from *WT* platelets. Thus, the combination of reduced (hemi-)ITAM-containing receptor expression combined with increased ITIM-containing receptor and Chk expression result in a reduction in platelet reactivity to specific substrates. This is better tolerated than pre-activated platelets that can trigger disseminated intravascular coagulation and death.

To circumvent masking effects of negative feedback mechanisms in *Csk KO* or *DKO* mice, we utilized *Csk^{AS}* mice. Inhibition of Csk^{AS} in platelets from these mice, resulted in significantly reduced phosphorylation of the C-terminal inhibitory tyrosine residues of SFKs, and increased SFK and Syk activity, similar to that reported in immune cells.^{39,57,67} However, inhibition of Csk^{AS} in platelets failed to have an effect on platelet aggregation. This can be partially explained by increased formation of the G6b-B-Shp1-Shp2 complex, counter-acting the effect of increased SFK activity on Syk activation.^{25,28} Our findings also suggest that Csk

plays little role in attenuating GPVI and CLEC-2 signaling once initiated, mainly providing a break prior to ligand engagement and receptor clustering to prevent unwanted signaling by the receptors. This was not however the case for $\alpha\text{IIb}\beta 3$ -mediated platelet spreading on fibrinogen, which was enhanced either in the absence of Csk or following inhibition of Csk^{AS}, suggesting Csk differentially regulates integrin and ITAM-containing receptor signaling. Previous work from our group suggests that G6b-B facilitates rather than inhibits integrin-mediated responses in platelets and MKs,²⁸ thus upregulation of the G6b-B-Shp1-Shp2 complex is predicted to enhance rather than attenuate platelet spreading, as observed.

In addition to filling a major gap in our knowledge of how SFKs are regulated in the MK lineage, findings from this study are also clinically relevant, demonstrating for the first time that either increased or decreased platelet SFK activity can lead to reduced thrombus formation and bleeding. Our study highlights, that loss of an inhibitor of platelet activation, such as Csk, can lead to paradoxical bleeding due to the robust negative feedback mechanisms that get activated. This may provide important mechanistic insights into the phenomenon of early platelet dysfunction observed in severe trauma patients displaying 'exhausted' platelets,⁶⁸ showing reduced thrombus formation on collagen.⁶⁹ Our findings that platelets in the circulation can undergo cell-intrinsic changes, leading to downregulation of activation pathways and upregulation of inhibitory mechanisms may contribute to platelet dysfunction in trauma patients. Moreover, certain tyrosine kinase inhibitors used to treat cancer patients inhibit SFKs or Csk and are accompanied with bleeding side-effects. Accordingly, the increased bleeding risk associated with use of the Bcr-Abl inhibitor Dasatinib is ascribed to its off-target effects on platelet SFKs.^{70,71} Similarly, bleeding-side effects of the Btk inhibitor Ibrutinib may be partially explained by its potent off-target effect on Csk.^{72,73} Based on our findings, CD148 is an attractive anti-thrombotic drug target, inhibition of which should attenuate, but not completely block any of the main platelet

activation pathways and have minimal bleeding side-effects. Tyrosine phosphatases are increasingly considered to be druggable,⁷⁴ supported by the recent development of the highly specific Shp2 inhibitor SHP099,⁷⁵ thus targeting CD148 is not inconceivable.

Acknowledgements

JM and ZN are British Heart Foundation (BHF) postdoctoral research associates, AM is a BHF Intermediate Basic Science Research Fellow (FS/15/58/31784) and YAS is a BHF Senior Basic Science Research Fellow (FS/13/1/29894). RA was supported by the government of the Sultanate of Oman. This work was supported by BHF Project Grant PG/11/108/29237 and Programme Grant RG/15/13/31673. We thank Timo Vögtle for critical reading of the manuscript and invaluable discussions, Quadrantech Diagnostics for providing the T-TAS device, and all members of the Birmingham Biomedical Sciences Unit for maintenance of mouse colonies.

Authorship Contributions

Conceptualization: JM, ZN, AM and YAS; Methodology: JM, ZN, JWMH, AM and YAS; Formal Analysis: JM, ZN, CWS, RA, JPG, BMET, GEJ, AM and YAS; Investigation: JM, ZN, GDN, CWS, MJG, RA, JPG, SH, LB, JNC, LT, MJEK, PH, JWMH, AM and YAS; Resources: YAS; Mouse models and reagents: AT and AW; Equipment and expertise: PH; Writing: JM, ZN, AM, and YAS; Revising and editing: JM, ZN, JWMH, GEJ, AW, AM and YAS; Supervision: AM and YAS

Conflict of Interest Disclosures

The authors declare no competing financial interests.

References

1. Golden A, Nemeth SP, Brugge JS. Blood platelets express high levels of the pp60c-src-specific tyrosine kinase activity. *Proc Natl Acad Sci U S A*. 1986;83(4):852-856.
2. Senis YA, Mazharian A, Mori J. Src family kinases: at the forefront of platelet activation. *Blood*. 2014;124(13):2013-2024.
3. Burkhardt JM, Vaudel M, Gambaryan S, et al. The first comprehensive and quantitative analysis of human platelet protein composition allows the comparative analysis of structural and functional pathways. *Blood*. 2012;120(15):e73-82.
4. Zeiler M, Moser M, Mann M. Copy number analysis of the murine platelet proteome spanning the complete abundance range. *Mol Cell Proteomics*. 2014;13(12):3435-3445.
5. Ezumi Y, Shindoh K, Tsuji M, Takayama H. Physical and functional association of the Src family kinases Fyn and Lyn with the collagen receptor glycoprotein VI-Fc receptor gamma chain complex on human platelets. *J Exp Med*. 1998;188(2):267-276.
6. Quek LS, Pasquet JM, Hers I, et al. Fyn and Lyn phosphorylate the Fc receptor gamma chain downstream of glycoprotein VI in murine platelets, and Lyn regulates a novel feedback pathway. *Blood*. 2000;96(13):4246-4253.
7. Arias-Salgado EG, Lizano S, Sarkar S, Brugge JS, Ginsberg MH, Shattil SJ. Src kinase activation by direct interaction with the integrin beta cytoplasmic domain. *Proc Natl Acad Sci U S A*. 2003;100(23):13298-13302.
8. Reddy KB, Smith DM, Plow EF. Analysis of Fyn function in hemostasis and alphaIIb beta3-integrin signaling. *J Cell Sci*. 2008;121(Pt 10):1641-1648.
9. Schmaier AA, Zou Z, Kazlauskas A, et al. Molecular priming of Lyn by GPVI enables an immune receptor to adopt a hemostatic role. *Proc Natl Acad Sci U S A*. 2009.
10. Maxwell MJ, Yuan Y, Anderson KE, Hibbs ML, Salem HH, Jackson SP. SHIP1 and Lyn Kinase Negatively Regulate Integrin alpha IIb beta 3 signaling in platelets. *J Biol Chem*. 2004;279(31):32196-32204.
11. Falati S, Edmead CE, Poole AW. Glycoprotein Ib-V-IX, a receptor for von Willebrand factor, couples physically and functionally to the Fc receptor gamma-chain, Fyn, and Lyn to activate human platelets. *Blood*. 1999;94(5):1648-1656.
12. Ozaki Y, Asazuma N, Suzuki-Inoue K, Berndt MC. Platelet GPIb-IX-V-dependent signaling. *J Thromb Haemost*. 2005;3(8):1745-1751.
13. Suzuki-Inoue K, Tulasne D, Shen Y, et al. Association of Fyn and Lyn with the proline-rich domain of glycoprotein VI regulates intracellular signaling. *J Biol Chem*. 2002;277(24):21561-21566.
14. Fox JE, Lipfert L, Clark EA, Reynolds CC, Austin CD, Brugge JS. On the role of the platelet membrane skeleton in mediating signal transduction. Association of GP IIb-IIIa, pp60c-src, pp62c-yes, and the p21ras GTPase-activating protein with the membrane skeleton. *J Biol Chem*. 1993;268(34):25973-25984.
15. Obergfell A, Eto K, Mocsai A, et al. Coordinate interactions of Csk, Src, and Syk kinases with [alpha]IIb[beta]3 initiate integrin signaling to the cytoskeleton. *J Cell Biol*. 2002;157(2):265-275.

16. Murugappan S, Shankar H, Bhamidipati S, Dorsam RT, Jin J, Kunapuli SP. Molecular mechanism and functional implications of thrombin-mediated tyrosine phosphorylation of PKCdelta in platelets. *Blood*. 2005;106(2):550-557.
17. Li Z, Zhang G, Liu J, et al. An important role of the SRC family kinase Lyn in stimulating platelet granule secretion. *J Biol Chem*. 2010;285(17):12559-12570.
18. Kim S, Kunapuli SP. Negative regulation of Gq-mediated pathways in platelets by G(12/13) pathways through Fyn kinase. *J Biol Chem*. 2011;286(27):24170-24179.
19. Xiang B, Zhang G, Stefanini L, et al. The Src family kinases and protein kinase C synergize to mediate Gq-dependent platelet activation. *J Biol Chem*. 2012;287(49):41277-41287.
20. Hardy AR, Jones ML, Mundell SJ, Poole AW. Reciprocal cross-talk between P2Y1 and P2Y12 receptors at the level of calcium signaling in human platelets. *Blood*. 2004;104(6):1745-1752.
21. Nash CA, Severin S, Dawood BB, et al. Src family kinases are essential for primary aggregation by G(i) -coupled receptors. *J Thromb Haemost*. 2010;8(10):2273-2282.
22. Poole A, Gibbins JM, Turner M, et al. The Fc receptor gamma-chain and the tyrosine kinase Syk are essential for activation of mouse platelets by collagen. *Embo J*. 1997;16(9):2333-2341.
23. Coxon CH, Geer MJ, Senis YA. ITIM receptors: more than just inhibitors of platelet activation. *Blood*. 2017;129(26):3407-3418.
24. Hua CT, Gamble JR, Vadas MA, Jackson DE. Recruitment and activation of SHP-1 protein-tyrosine phosphatase by human platelet endothelial cell adhesion molecule-1 (PECAM-1). Identification of immunoreceptor tyrosine-based inhibitory motif-like binding motifs and substrates. *J Biol Chem*. 1998;273(43):28332-28340.
25. Mazharian A, Mori J, Wang YJ, et al. Megakaryocyte-specific deletion of the protein-tyrosine phosphatases Shp1 and Shp2 causes abnormal megakaryocyte development, platelet production, and function. *Blood*. 2013;121(20):4205-4220.
26. Jackson DE, Ward CM, Wang R, Newman PJ. The protein-tyrosine phosphatase SHP-2 binds platelet/endothelial cell adhesion molecule-1 (PECAM-1) and forms a distinct signaling complex during platelet aggregation. Evidence for a mechanistic link between PECAM-1- and integrin-mediated cellular signaling. *J Biol Chem*. 1997;272(11):6986-6993.
27. Falati S, Patil S, Gross PL, et al. Platelet PECAM-1 inhibits thrombus formation in vivo. *Blood*. 2006;107(2):535-541.
28. Mazharian A, Wang YJ, Mori J, et al. Mice lacking the ITIM-containing receptor G6b-B exhibit macrothrombocytopenia and aberrant platelet function. *Sci Signal*. 2012;5(248):ra78.
29. Hermiston ML, Xu Z, Weiss A. CD45: a critical regulator of signaling thresholds in immune cells. *Annu Rev Immunol*. 2003;21:107-137.
30. Senis YA, Tomlinson MG, Ellison S, et al. The tyrosine phosphatase CD148 is an essential positive regulator of platelet activation and thrombosis. *Blood*. 2009;113(20):4942-4954.
31. Ellison S, Mori J, Barr AJ, Senis YA. CD148 enhances platelet responsiveness to collagen by maintaining a pool of active Src family kinases. *J Thromb Haemost*. 2010;8(7):1575-1583.

32. Mori J, Wang YJ, Ellison S, et al. Dominant role of the protein-tyrosine phosphatase CD148 in regulating platelet activation relative to protein-tyrosine phosphatase-1B. *Arterioscler Thromb Vasc Biol.* 2012;32(12):2956-2965.
33. Roskoski R, Jr. Src kinase regulation by phosphorylation and dephosphorylation. *Biochem Biophys Res Commun.* 2005;331(1):1-14.
34. Nada S, Okada M, MacAuley A, Cooper JA, Nakagawa H. Cloning of a complementary DNA for a protein-tyrosine kinase that specifically phosphorylates a negative regulatory site of p60c-src. *Nature.* 1991;351(6321):69-72.
35. Davidson D, Chow LM, Veillette A. Chk, a Csk family tyrosine protein kinase, exhibits Csk-like activity in fibroblasts, but not in an antigen-specific T-cell line. *J Biol Chem.* 1997;272(2):1355-1362.
36. Schmedt C, Saijo K, Niidome T, Kuhn R, Aizawa S, Tarakhovsky A. Csk controls antigen receptor-mediated development and selection of T-lineage cells. *Nature.* 1998;394(6696):901-904.
37. Katsumoto TR, Kudo M, Chen C, et al. The phosphatase CD148 promotes airway hyperresponsiveness through SRC family kinases. *J Clin Invest.* 2013;123(5):2037-2048.
38. Tiedt R, Schomber T, Hao-Shen H, Skoda RC. Pf4-Cre transgenic mice allow the generation of lineage-restricted gene knockouts for studying megakaryocyte and platelet function in vivo. *Blood.* 2007;109(4):1503-1506.
39. Tan YX, Manz BN, Freedman TS, Zhang C, Shokat KM, Weiss A. Inhibition of the kinase Csk in thymocytes reveals a requirement for actin remodeling in the initiation of full TCR signaling. *Nat Immunol.* 2014;15(2):186-194.
40. Pearce AC, Senis YA, Billadeau DD, Turner M, Watson SP, Vigorito E. Vav1 and vav3 have critical but redundant roles in mediating platelet activation by collagen. *J Biol Chem.* 2004;279(52):53955-53962.
41. Ohlmann P, Eckly A, Freund M, Cazenave JP, Offermanns S, Gachet C. ADP induces partial platelet aggregation without shape change and potentiates collagen-induced aggregation in the absence of Galphag. *Blood.* 2000;96(6):2134-2139.
42. Smith CW, Thomas SG, Raslan Z, et al. Mice Lacking the Inhibitory Collagen Receptor LAIR-1 Exhibit a Mild Thrombocytosis and Hyperactive Platelets. *Arterioscler Thromb Vasc Biol.* 2017;37(5):823-835.
43. Pertuy F, Aguilar A, Strassel C, et al. Broader expression of the mouse platelet factor 4-cre transgene beyond the megakaryocyte lineage. *J Thromb Haemost.* 2015;13(1):115-125.
44. Spangrude GJ, Lewandowski D, Martelli F, et al. P-Selectin Sustains Extramedullary Hematopoiesis in the Gata1 low Model of Myelofibrosis. *Stem Cells.* 2016;34(1):67-82.
45. Schaff M, Tang C, Maurer E, et al. Integrin alpha6beta1 is the main receptor for vascular laminins and plays a role in platelet adhesion, activation, and arterial thrombosis. *Circulation.* 2013;128(5):541-552.
46. de Witt SM, Swieringa F, Cavill R, et al. Identification of platelet function defects by multi-parameter assessment of thrombus formation. *Nat Commun.* 2014;5:4257.

47. Nika K, Soldani C, Salek M, et al. Constitutively active Lck kinase in T cells drives antigen receptor signal transduction. *Immunity*. 2010;32(6):766-777.
48. Severin S, Nash CA, Mori J, et al. Distinct and overlapping functional roles of Src family kinases in mouse platelets. *J Thromb Haemost*. 2012;10(8):1631-1645.
49. Arias-Salgado EG, Lizano S, Shattil SJ, Ginsberg MH. Specification of the direction of adhesive signaling by the integrin beta cytoplasmic domain. *J Biol Chem*. 2005;280(33):29699-29707.
50. Pera IL, Iuliano R, Florio T, et al. The rat tyrosine phosphatase eta increases cell adhesion by activating c-Src through dephosphorylation of its inhibitory phosphotyrosine residue. *Oncogene*. 2005;24(19):3187-3195.
51. Stepanek O, Kalina T, Draber P, et al. Regulation of Src family kinases involved in T cell receptor signaling by protein-tyrosine phosphatase CD148. *J Biol Chem*. 2011;286(25):22101-22112.
52. McNeill L, Salmond RJ, Cooper JC, et al. The differential regulation of Lck kinase phosphorylation sites by CD45 is critical for T cell receptor signaling responses. *Immunity*. 2007;27(3):425-437.
53. Zikherman J, Jenne C, Watson S, et al. CD45-Csk phosphatase-kinase titration uncouples basal and inducible T cell receptor signaling during thymic development. *Immunity*. 2010;32(3):342-354.
54. Lin J, Weiss A. The tyrosine phosphatase CD148 is excluded from the immunologic synapse and down-regulates prolonged T cell signaling. *J Cell Biol*. 2003;162(4):673-682.
55. Davis SJ, van der Merwe PA. The kinetic-segregation model: TCR triggering and beyond. *Nat Immunol*. 2006;7(8):803-809.
56. Schmedt C, Tarakhovsky A. Autonomous maturation of alpha/beta T lineage cells in the absence of COOH-terminal Src kinase (Csk). *J Exp Med*. 2001;193(7):815-826.
57. Freedman TS, Tan YX, Skrzypczynska KM, et al. LynA regulates an inflammation-sensitive signaling checkpoint in macrophages. *Elife*. 2015;4.
58. Furukawa T, Itoh M, Krueger NX, Streuli M, Saito H. Specific interaction of the CD45 protein-tyrosine phosphatase with tyrosine-phosphorylated CD3 zeta chain. *Proc Natl Acad Sci U S A*. 1994;91(23):10928-10932.
59. Kashio N, Matsumoto W, Parker S, Rothstein DM. The second domain of the CD45 protein tyrosine phosphatase is critical for interleukin-2 secretion and substrate recruitment of TCR-zeta in vivo. *J Biol Chem*. 1998;273(50):33856-33863.
60. Isakov N, Wange RL, Burgess WH, Watts JD, Aebersold R, Samelson LE. ZAP-70 binding specificity to T cell receptor tyrosine-based activation motifs: the tandem SH2 domains of ZAP-70 bind distinct tyrosine-based activation motifs with varying affinity. *J Exp Med*. 1995;181(1):375-380.
61. Ottinger EA, Botfield MC, Shoelson SE. Tandem SH2 domains confer high specificity in tyrosine kinase signaling. *J Biol Chem*. 1998;273(2):729-735.
62. Iwashima M, Irving BA, van Oers NS, Chan AC, Weiss A. Sequential interactions of the TCR with two distinct cytoplasmic tyrosine kinases. *Science*. 1994;263(5150):1136-1139.

63. Ashe JM, Wiest DL, Abe R, Singer A. ZAP-70 protein promotes tyrosine phosphorylation of T cell receptor signaling motifs (ITAMs) in immature CD4(+)8(+) thymocytes with limiting p56(lck). *J Exp Med*. 1999;189(7):1163-1168.
64. Qian D, Mollenauer MN, Weiss A. Dominant-negative zeta-associated protein 70 inhibits T cell antigen receptor signaling. *J Exp Med*. 1996;183(2):611-620.
65. Coxon CH, Sadler AJ, Huo J, Campbell RD. An investigation of hierarchical protein recruitment to the inhibitory platelet receptor, G6B-b. *PLoS One*. 2012;7(11):e49543.
66. Hirao A, Hamaguchi I, Suda T, Yamaguchi N. Translocation of the Csk homologous kinase (Chk/Hyl) controls activity of CD36-anchored Lyn tyrosine kinase in thrombin-stimulated platelets. *Embo J*. 1997;16(9):2342-2351.
67. Manz BN, Tan YX, Courtney AH, et al. Small molecule inhibition of Csk alters affinity recognition by T cells. *Elife*. 2015;4.
68. Wohlaer MV, Moore EE, Thomas S, et al. Early platelet dysfunction: an unrecognized role in the acute coagulopathy of trauma. *J Am Coll Surg*. 2012;214(5):739-746.
69. Li R, Elmongy H, Sims C, Diamond SL. Ex vivo recapitulation of trauma-induced coagulopathy and preliminary assessment of trauma patient platelet function under flow using microfluidic technology. *J Trauma Acute Care Surg*. 2016;80(3):440-449.
70. Quintas-Cardama A, Han X, Kantarjian H, Cortes J. Tyrosine kinase inhibitor-induced platelet dysfunction in patients with chronic myeloid leukemia. *Blood*. 2009;114(2):261-263.
71. Gratacap MP, Martin V, Valera MC, et al. The new tyrosine-kinase inhibitor and anticancer drug dasatinib reversibly affects platelet activation in vitro and in vivo. *Blood*. 2009;114(9):1884-1892.
72. Levade M, David E, Garcia C, et al. Ibrutinib treatment affects collagen and von Willebrand factor-dependent platelet functions. *Blood*. 2014;124(26):3991-3995.
73. Honigberg LA, Smith AM, Sirisawad M, et al. The Bruton tyrosine kinase inhibitor PCI-32765 blocks B-cell activation and is efficacious in models of autoimmune disease and B-cell malignancy. *Proc Natl Acad Sci U S A*. 2010;107(29):13075-13080.
74. Tautz L, Senis YA, Oury C, Rahmouni S. Perspective: Tyrosine phosphatases as novel targets for antiplatelet therapy. *Bioorg Med Chem*. 2015;23(12):2786-2797.
75. Chen YN, LaMarche MJ, Chan HM, et al. Allosteric inhibition of SHP2 phosphatase inhibits cancers driven by receptor tyrosine kinases. *Nature*. 2016;535(7610):148-152.
76. Nieminen P LH, Vähäkangas K, Huusko A, Rautio A. Standardised regression coefficient as an effect size index in summarising findings in epidemiological studies. *Epidemiology Biostatistics and Public Health*. 2013;Volume 10(4):e8854.

Table 1. Summary of *Csk KO*, *CD148 KO* and *DKO* mouse phenotypes.

Phenotype	Genotype		
	<i>Csk KO</i>	<i>CD148 KO</i>	<i>DKO</i>
Bleeding	↑	—	↑↑
Thrombosis			
<i>In vivo</i>			
Laser injury	↓	↓	↓↓
FeCl ₃ injury	↓↓	↓	↓
<i>Ex vivo</i>			
T-TAS – collagen	↓	↓	↓↓
– collagen, tissue factor	—	—	—
Flow chamber – collagen	↓	↓	↓↓
– vWF-BP, laminin, rhodocytin	↓	↓	↓
Platelet count	↓	—	—
Platelet receptors			
Expression			
GPVI-FcRγ-chain	↓↓	↓	↓↓↓
CLEC-2	↓	—	↓↓
G6b-B	↑↑	—	↑↑
Phosphorylation			
G6b-B	↑↑	—	↑↑↑
SFK activity	↑	↓	↑↑

↑ upregulated, ↓ downregulated, — normal

Figure Legends

Figure 1. Aberrant platelet production and platelet receptor expressions in *Csk KO* mice.

(A) Platelet lysates were blotted for the indicated proteins. (B) Platelet counts. (C) Percentage of reticulated platelets determined by thiazole orange⁺ α IIb⁺ cells in blood. See also Figure S2A. (D) i) Median fluorescence intensity (MFI) measured in α IIb⁺ cells alone or α IIb⁺ cells co-stained for the indicated proteins in blood, n = 5-6 mice/genotype. See also Figure S3. ii) Platelet lysates were blotted for the indicated proteins. (E) i) Platelet counts of pre-/post-injection of anti-GPIb α antibody (1.5 μ g/g of body weight). ii) The rate of platelet recovery determined by a proportionate slope from linear trend lines between day 3 to 7 from (i), n = 7-8 mice/genotype. See also Figures S4-6.

Asterisks refer to significant difference compared to *WT*. **P* < 0.05, ****P* < 0.001, one-way ANOVA with Tukey's test (B-D), or one-way ANOVA with Dunnett's test; vs *WT* (E), mean \pm SEM.

Figure 2. Increased bleeding and defective thrombus formation in *Csk KO* and *DKO* mice.

(A) Hemostatic response was measured in tail bleeding assays by an excision of a 5-mm portion of the tail tip followed by the determination of lost blood/body weight (normalized blood loss), n = 11-57 mice/genotype. Tail bleeding assays were conducted in a double-blinded manner. (B) Laser injury-induced thrombus formation *in vivo*. Composite brightfield and fluorescence images of i) platelet accumulation (green) or ii) fibrin generation (red) in cremaster muscle arterioles monitored by DyLight488-labeled anti-GPIIb β antibody (0.1 μ g/g of body weight) or Alexa-Fluor 647-labeled anti-fibrin antibody (0.2 μ g/g of body

weight) signal, respectively by confocal intravital microscopy (scale bar: 10 μm). Each curve represents the median integrated fluorescence intensity of iii) platelets or iv) fibrin in relative fluorescence units (RFU), $n = 25\text{-}37$, in 5 mice/genotype. See also Videos 1 and 2. (C) Ferric chloride injury-induced thrombus formation *in vivo*. Filter paper soaked in 10% ferric chloride was applied to carotid artery for 3 minutes. i) Representative fluorescence images of platelet accumulation (green) monitored by DyLight488-labeled anti-GPIIb β antibody (0.1 $\mu\text{g/g}$ of body weight) by confocal intravital microscopy (scale bar: 200 μm). ii) Each curve represents the median integrated thrombus fluorescence intensity in RFU. iii) Area under the curve (AUC) was measured, $n = 8\text{-}11$ mice per genotype. See also Video 3.

* $P < 0.05$, ** $p < 0.01$, *** $p < 0.001$, one-way ANOVA with Tukey's test; mean \pm SEM

Figure 3. Aberrant platelet functions in *Csk KO*, *CD148 KO* and *DKO* mice.

(A) i) 25 $\mu\text{g/ml}$ hirudin-treated blood was perfused with collagen-coated chip at 1000 s^{-1} for 10 minutes. Individual time-dependent flow pressure curves and total thrombogenicity (Area under the curve: AUC) were measured by the total thrombus-formation analysis system (T-TAS). ii) Blood treated with 3.2% sodium citrate, 12 mM CaCl_2 and 50 $\mu\text{g/ml}$ corn trypsin inhibitor was perfused on collagen plus tissue thromboplastin (tissue factor)-coated chip at 240 s^{-1} for 30 minutes. Individual time-dependent flow pressure curves, time to onset (T10), time to occlusion (T80), rate of thrombus growth (T10-80) and AUC were measured by T-TAS, $n = 4\text{-}9$ per genotype. (B) Blood treated with 5 Unit/ml Heparin, 40 μM PPACK and 50 Unit/ml Fragmin was perfused over microspots with indicated coatings; 100 $\mu\text{g/ml}$ collagen I, 12.5 $\mu\text{g/ml}$ vWF-binding peptide (vWF-BP), 50 $\mu\text{g/ml}$ laminin, 250 $\mu\text{g/ml}$ rhodocytin for 3.5 minutes at $1,000 \text{ s}^{-1}$. i) Representative bright field images with indicated surfaces. ii) Platelet deposition (% surface area coverage; SAC). iii) Multilayered thrombus

(% SAC) on collagen I surface. iv) Representative fluorescence images of P-selectin, integrin $\alpha\text{IIb}\beta 3$ activation (JON/A) and PS exposure (Annexin V) from collagen surface and v) % SAC of fluorescence images. vi) Heatmap of outcome parameters expressed as effect sizes per genotype;⁷⁶ P1, platelet deposition, P2, multilayered thrombus, P3, P-selectin, P4, JON/A, and P5, PS exposure from indicated surfaces. Scale bar: 10 μm . Experiments and analysis were performed in a double-blinded manner. % SAC was analyzed using Fiji programme, $n = 15$ images from 5 mice per genotype. (C) Representative platelet aggregation and ATP secretion traces in response to the indicated agonists in i-vi) washed platelets or vii) ADP-sensitive washed platelets. See also Figure S7. $n = 4-8$ mice/condition/genotype. (D) i) Representative differential interference contrast (DIC) microscopy images of resting (basal) and thrombin-stimulated (0.1 Unit/ml, 5 minutes) platelets spread on fibrinogen-coated cover-slips (100 $\mu\text{g/ml}$, 45 minutes, 37°C, scale bar: 5 μm). ii) Mean surface area of individual platelets quantified by ImageJ software, $n = 256$ platelets/condition/genotype. (E) Fibrin clot retraction was assessed 2 hours after the addition of 1 Unit/ml thrombin and 2 mM CaCl_2 to platelet-rich plasma. Clot volumes were expressed as a percentage of the initial volume of platelet-rich plasma, $n = 8$ per genotype. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, two-way (Bii, Bv, and D) or one-way (A, Biii, and E) ANOVA with Tukey's test, mean \pm SEM.

Figure 4. Csk and CD148 reciprocally regulate platelet SFKs.

(A) i) Representative electropherograms of capillary-based immunoassays on platelet lysates with the indicated antibodies and the quantification of peak areas, $n = 6$ mice/genotype. ii) Representative data from i) displayed as blots. See also Figure S8. (B) Model of SFK regulation in platelets. In *WT* platelets, SFKs are constrained in an inactive conformation by Csk, which phosphorylates the C-terminal inhibitory tyrosine residue. SFKs can be activated

by dephosphorylation of the inhibitory residue by CD148. SFKs *trans*-auto-phosphorylate each other at the activation-loop tyrosine residue and become fully active. CD148 can also dephosphorylate the activation-loop tyrosine leading to a decrease in SFK activity. In *Csk KO* platelets, CD148 dominates resulting in the loss of inhibitory phosphorylation and a net increase in SFK activity. In *CD148 KO* platelets, Csk dominates resulting in increased inhibitory phosphorylation, and markedly decreased activation-loop phosphorylation. In *DKO* platelets, the absence of both Csk and CD148 leads to a dramatic increase in SFK activity. The differential phosphorylation of SFKs in *Csk KO* and *DKO* platelets supports the hypothesis of CD148 dephosphorylating both the activation-loop and the C-terminal inhibitory tyrosine residues. (C) Platelet lysates were blotted for the indicated proteins.

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, repeated measures (RM) one-way ANOVA with Tukey's test, mean \pm SEM.

Figure 5. Csk and CD148 are critical for platelet activation signals.

(A) i) Lysates of basal and collagen-stimulated (30 μ g/ml, 90 seconds) platelets were blotted for p-Tyr and tubulin. ii-iii) Same lysates were analyzed by capillary-based immunoassays with the indicated antibodies. ii) Representative data displayed as blots. iii) Quantification of peak areas from Figure S9A, $n = 3-4$ mice/genotype. See also Figure S9A. (B) i-iii) Lysates of basal and CLEC-2 antibody-stimulated (10 μ g/ml, 5 minutes) platelets were analyzed as described in (A), $n = 3-4$ mice/genotype. See also Figure S9B. (C) i-iii) Lysates of non-adherent (NA) and adherent (Ad) platelets from fibrinogen-coated plates (100 μ g/ml, 45 minutes, 37°C) were analyzed as described in (A). See also Figure S9C. iv) Same lysates were blotted for Syk p-Tyr525/6, $n = 3-4$ mice/genotype. Differences in basal SFK p-Tyr418 between *WT* and *DKO* platelets in suspension (A-B) compared with non-adhered platelets (C), are due to differences in sample preparation, as demonstrated by a significant increase in Src

p-Tyr418 levels in non-adhered platelets incubated on fibrinogen-coated surface (Figure S9D).

(D) i) Increased G6b-B phosphorylation and assembly of the G6b-B-Shp1-Shp2 complex in *Csk KO* and *DKO* platelets. Lysates of basal and collagen-stimulated (30 μ g/ml, 90 seconds) platelets were immunoprecipitated with anti-G6b-B antibody and blotted for p-Tyr, G6b-B, Shp1 and Shp2. ii) The role of SFKs in activating and inhibitory pathways. Once a threshold level of SFK activity is reached, ITAM, integrin and ITIM signaling are triggered. The kinetics and order of activation depend upon the agonist and level of SFK activity.

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, RM two-way ANOVA with Tukey's test, mean \pm SEM.

Figure 6. Csk regulates ITAM-, ITIM- and integrin-dependent signaling in *Csk*^{AS} platelets.

(A-E) Platelets from *Csk*^{AS} mice were pre-treated with either DMSO or 3-IB-PP1 (10 μ M, 10 minutes). (A) i-iv) Representative aggregation and ATP secretion traces of platelets from *Csk*^{AS} mice in response to the indicated agonists, n = 3-4 mice/condition/genotype. (B) i) Representative DIC microscopy images of resting (basal) and thrombin-stimulated (0.1 Unit/ml, 5 minutes) platelets spread on fibrinogen-coated cover-slips (100 μ g/ml, 45 minutes, 37°C, scale bar: 5 μ m). ii) Mean surface area of individual platelets quantified by ImageJ software, n = 256 platelets/condition/genotype. (C and D) Lysates of platelets incubated with or without CRP (30 μ g/ml, C) or CLEC-2 antibody (10 μ g/ml, D) for indicated times were i) blotted for p-Tyr and actin, or ii-iii) analyzed by capillary-based immunoassays as described in Figure 5A, n = 3-4 mice/genotype. See also Figure S10A-B. (E) Increased G6b-B phosphorylation and assembly of the G6b-B-Shp1-Shp2 complex in collagen-stimulated *Csk*^{AS} platelets treated with 3-IB-PP1. Lysates of basal and collagen-stimulated (30 μ g/ml,

90 seconds) platelets were immunoprecipitated with anti-G6b-B antibody and blotted for p-Tyr, G6b-B, Shp1 and Shp2.

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ordinary (B) or RM (C and D) two-way ANOVA with Sidak's test, mean \pm SEM.

Figure 7. Regulation of platelet SFKs by the kinase Csk, and the phosphatase CD148.

(A) Csk and CD148 are the main regulators of SFK activity in platelets and impact on different platelet signaling pathways. Csk inhibits SFK activity, whereas CD148 activates SFKs but it can also inhibit SFK activity under as yet undefined conditions. In turn, SFKs regulate ITAM, integrin, ITIM and P2Y₁₂ receptor signaling pathways. See also Figures 4B and 5Dii. (B) Post-activated platelets. Platelets possess remarkable positive-feedback pathways (e.g. ADP, TXA₂), which further enhance platelet activation. Platelet activation is followed by receptor proteolysis or internalization, *de novo* protein synthesis and downregulation of tyrosine phosphorylation pathways by ITIM-containing receptors and phosphatases. *Csk KO* and *DKO* platelets have increased SFK activity which results in reduced expression of the platelet activating receptors GPVI and CLEC-2, increased expression of the ITIM-containing receptor G6b-B and increased ITIM signaling suggesting that these platelets exist in a 'post-activated' state.

Figure 1

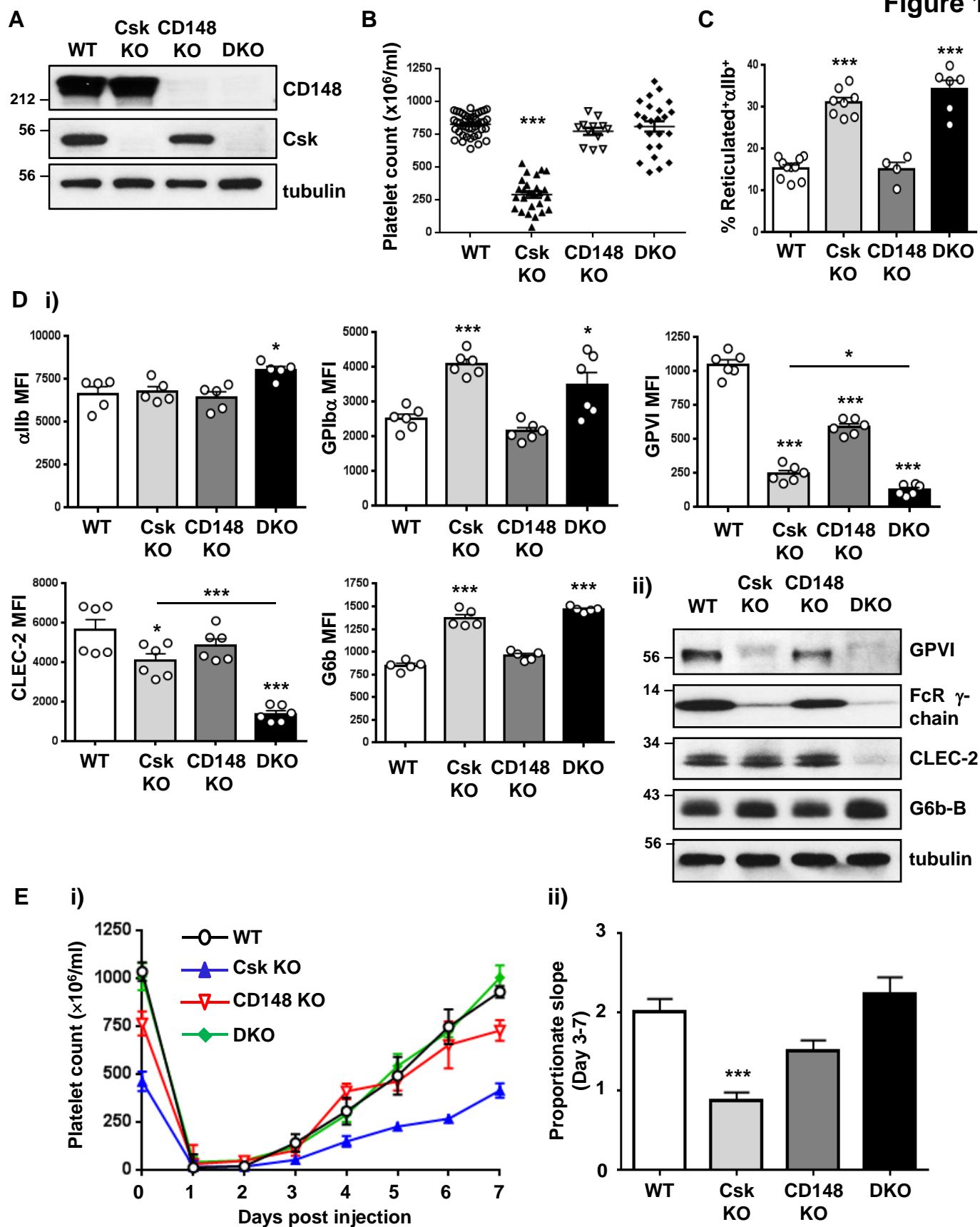
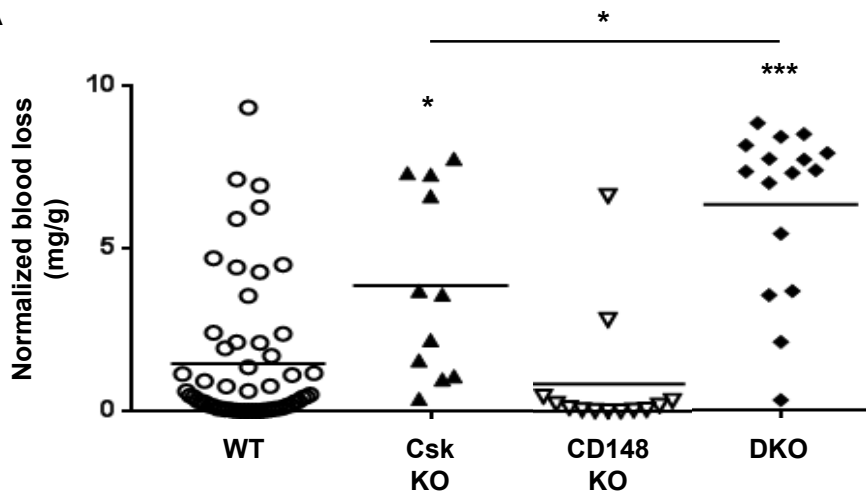
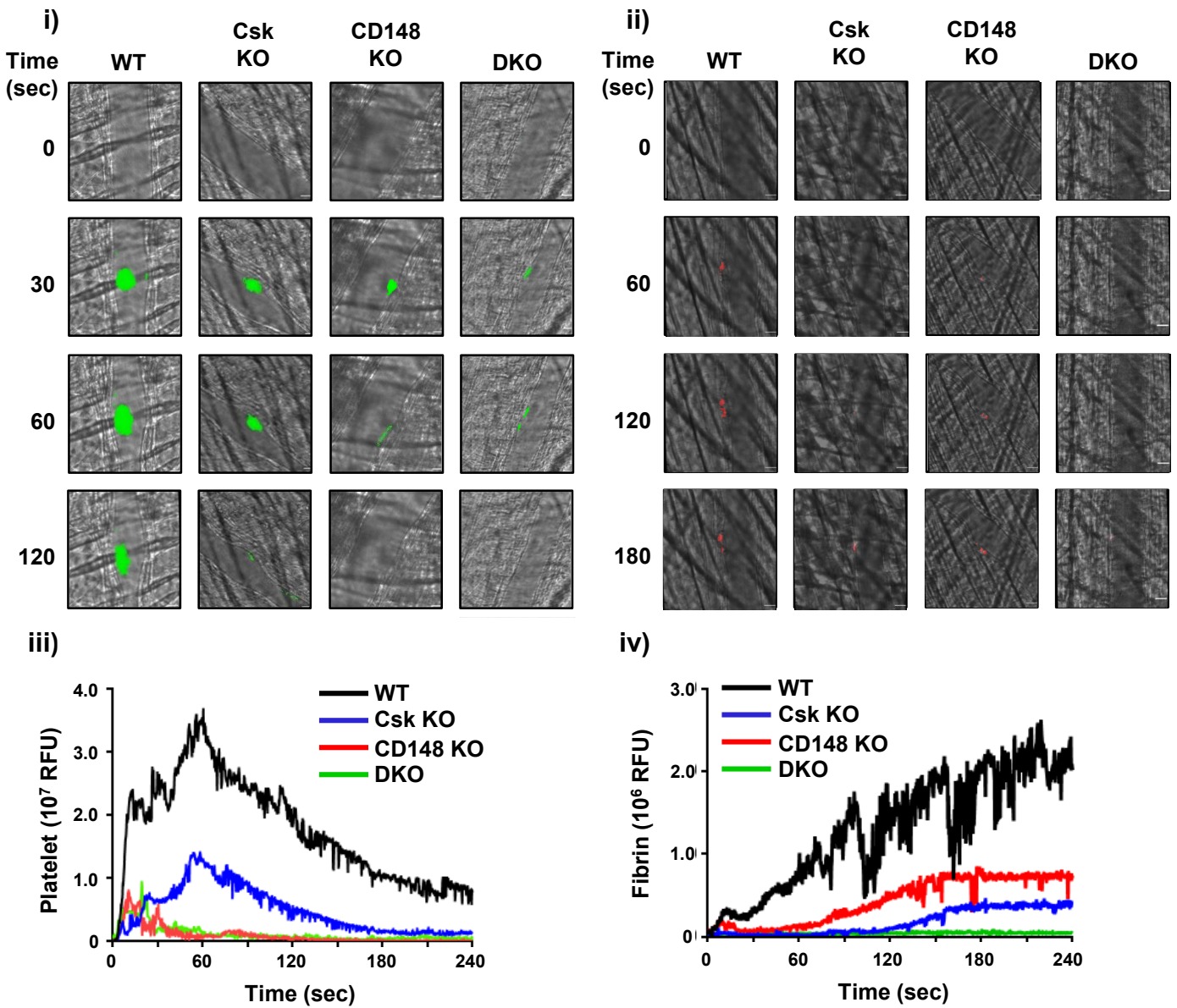


Figure 2

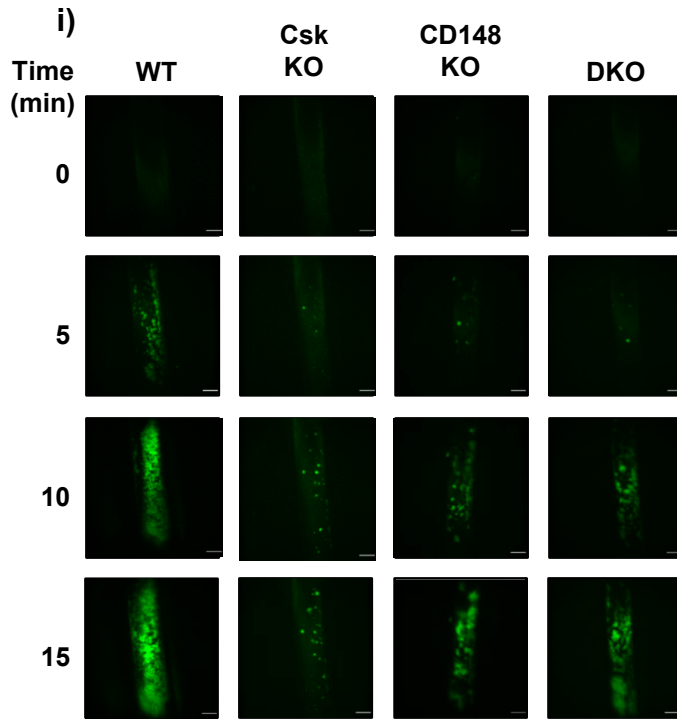
A



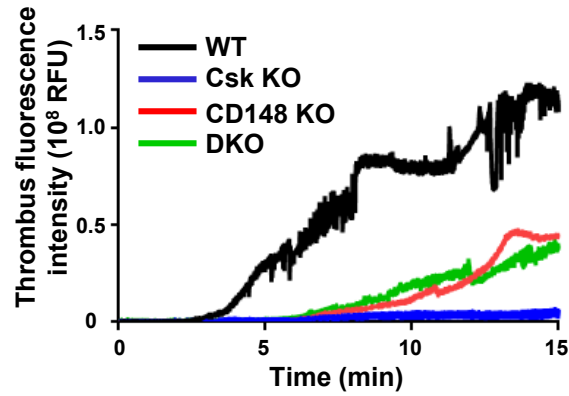
B



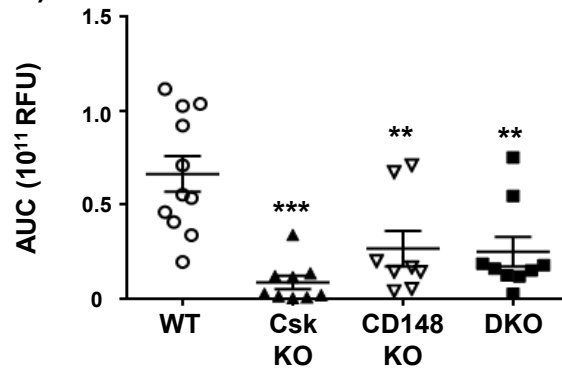
C



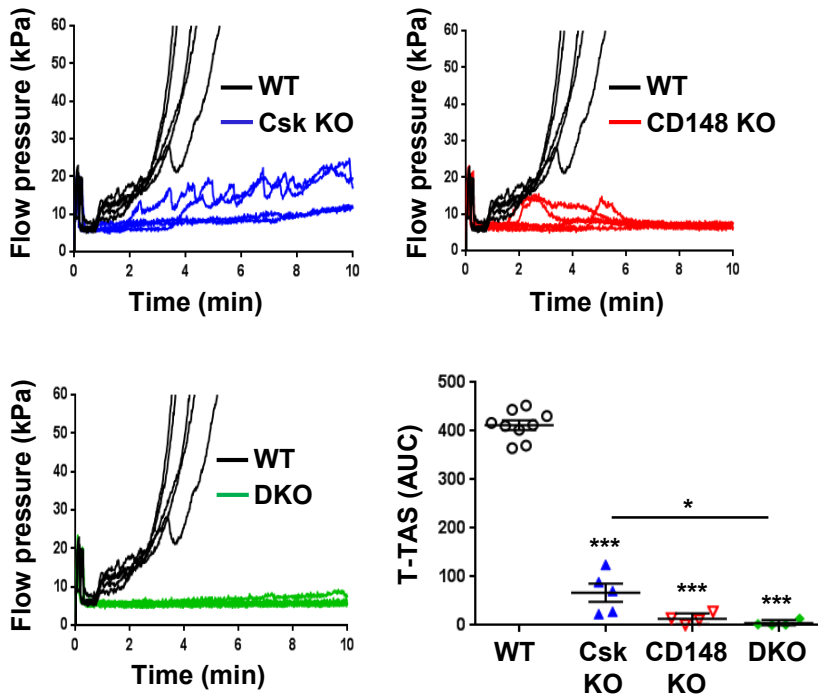
ii)



iii)



A i) collagen



ii) collagen + tissue factor

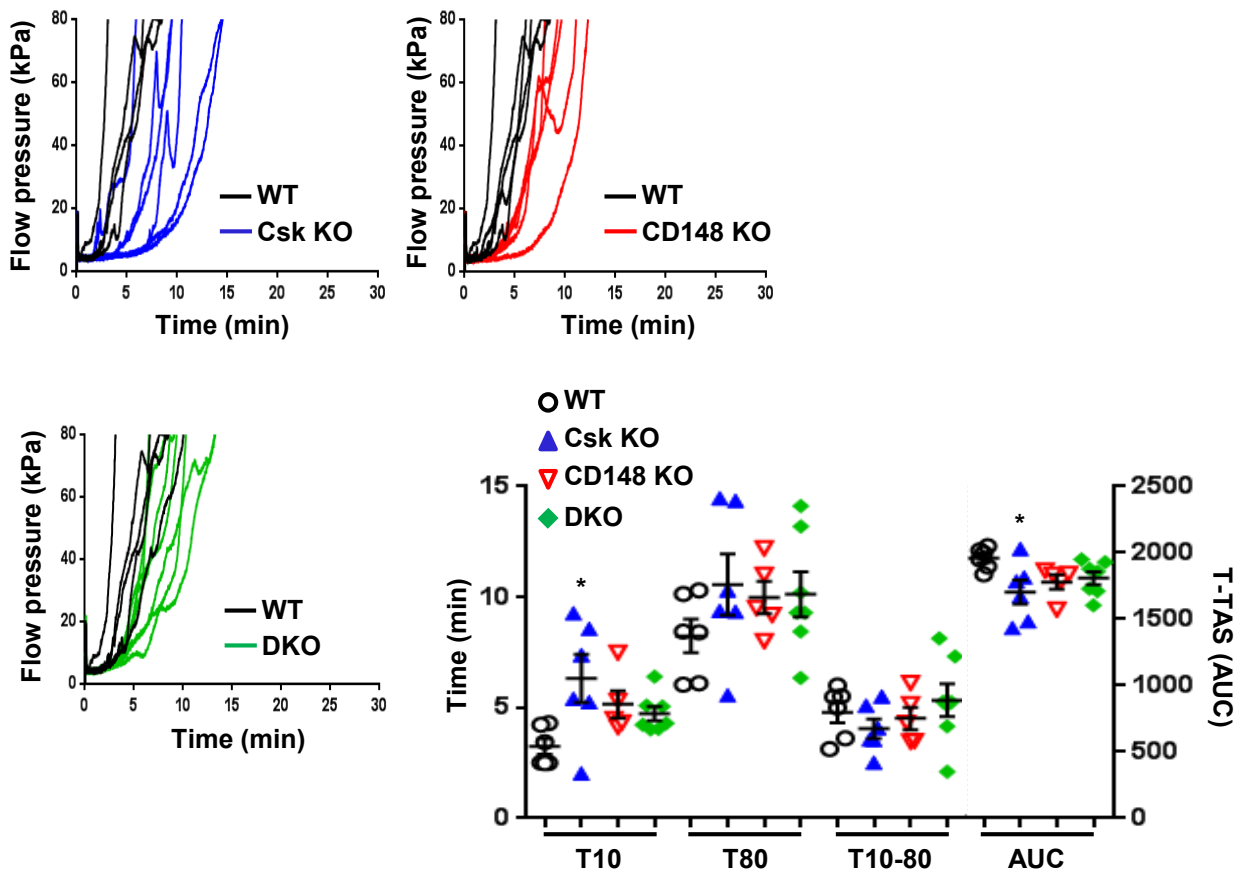


Figure 3

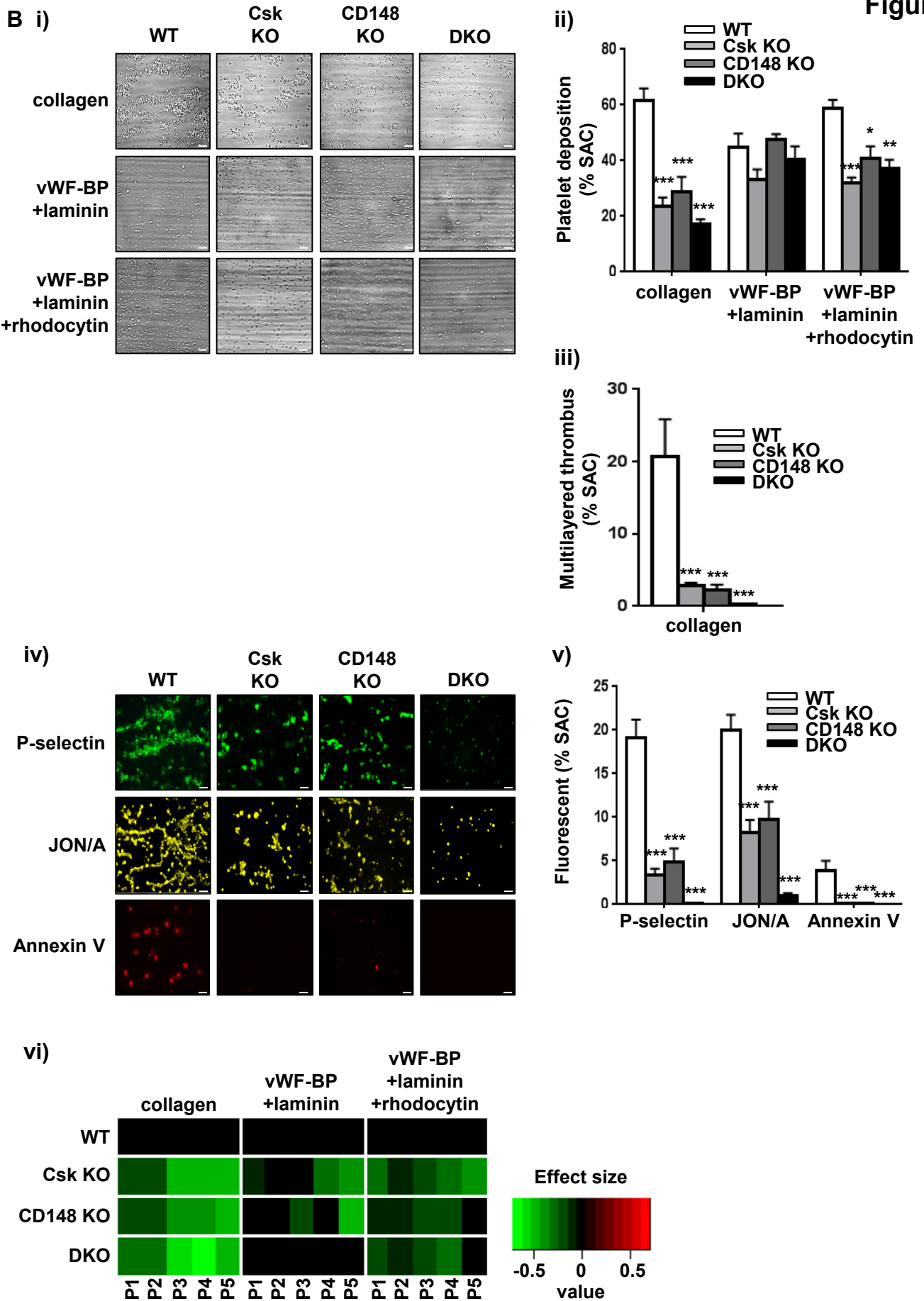
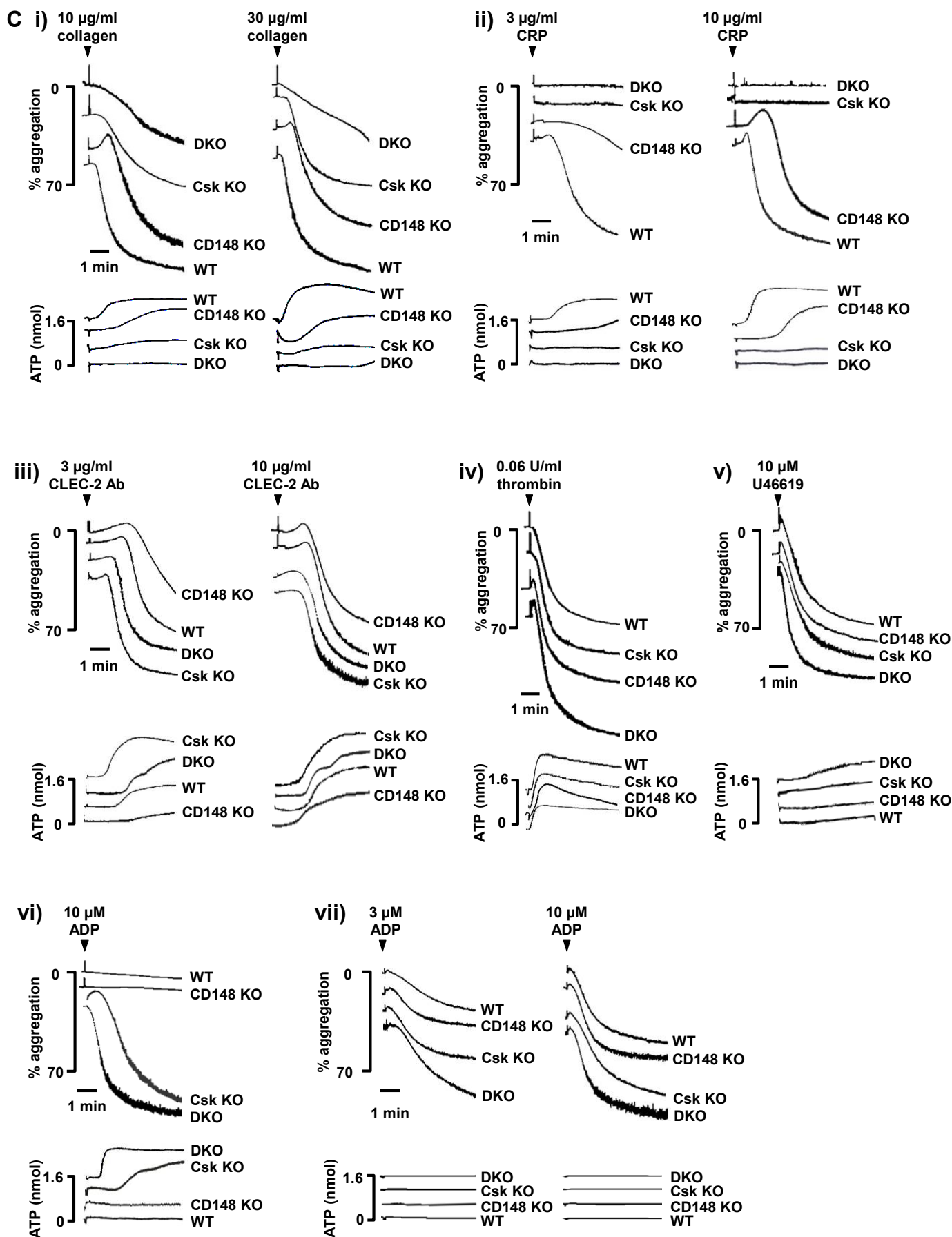


Figure 3



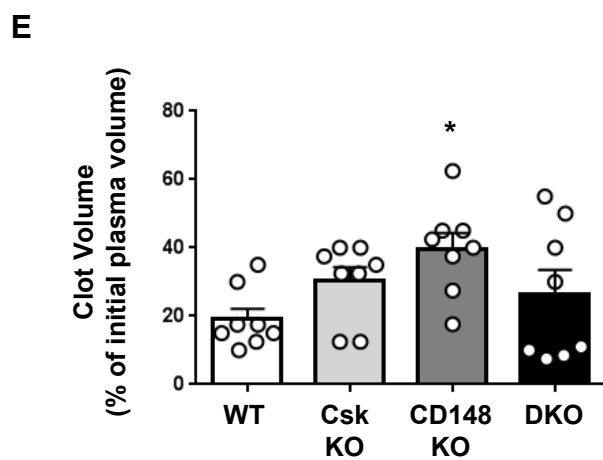
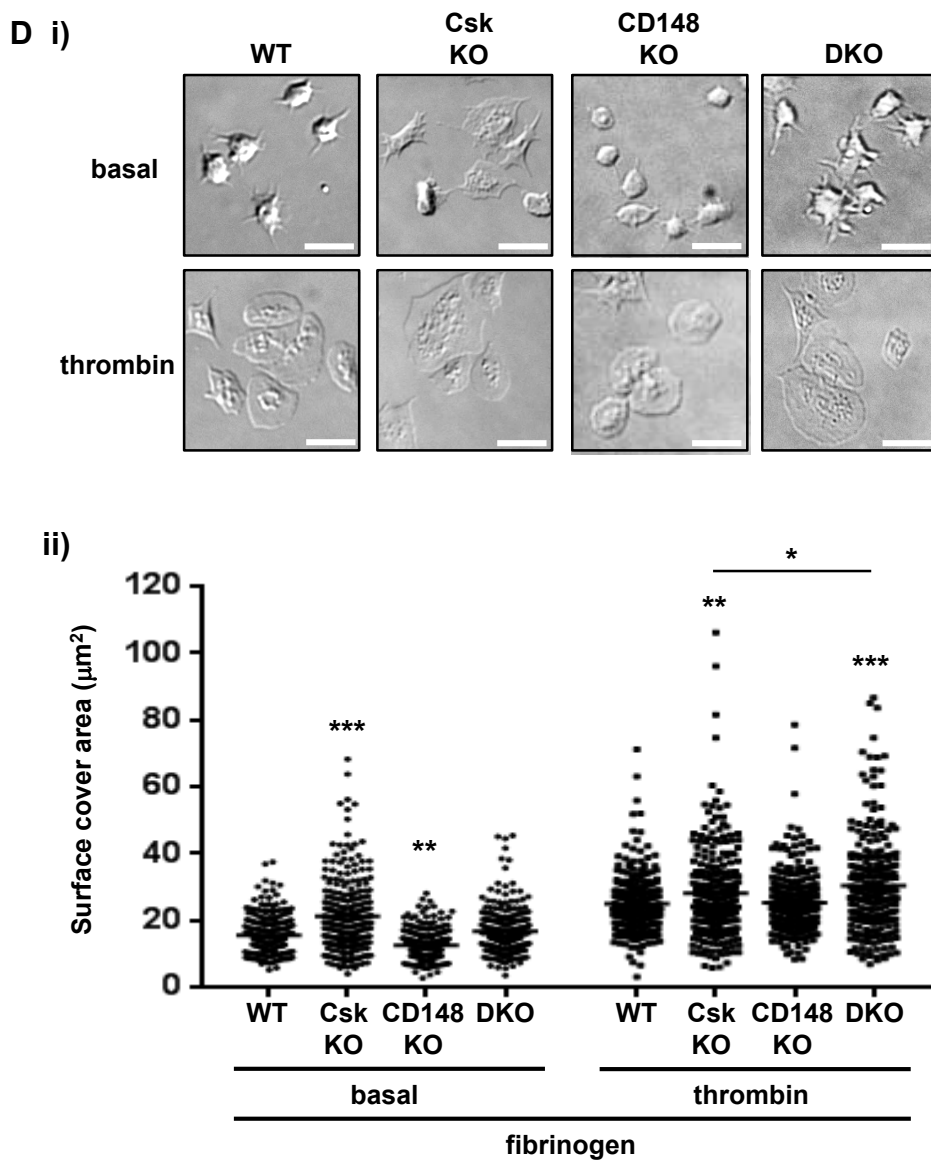
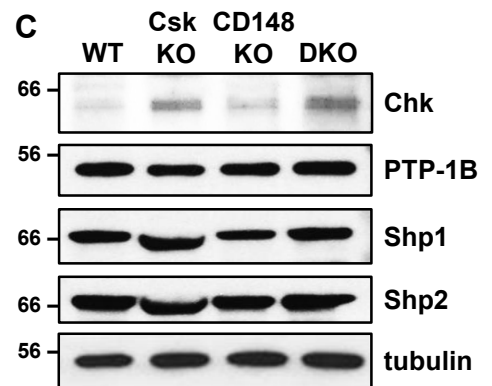
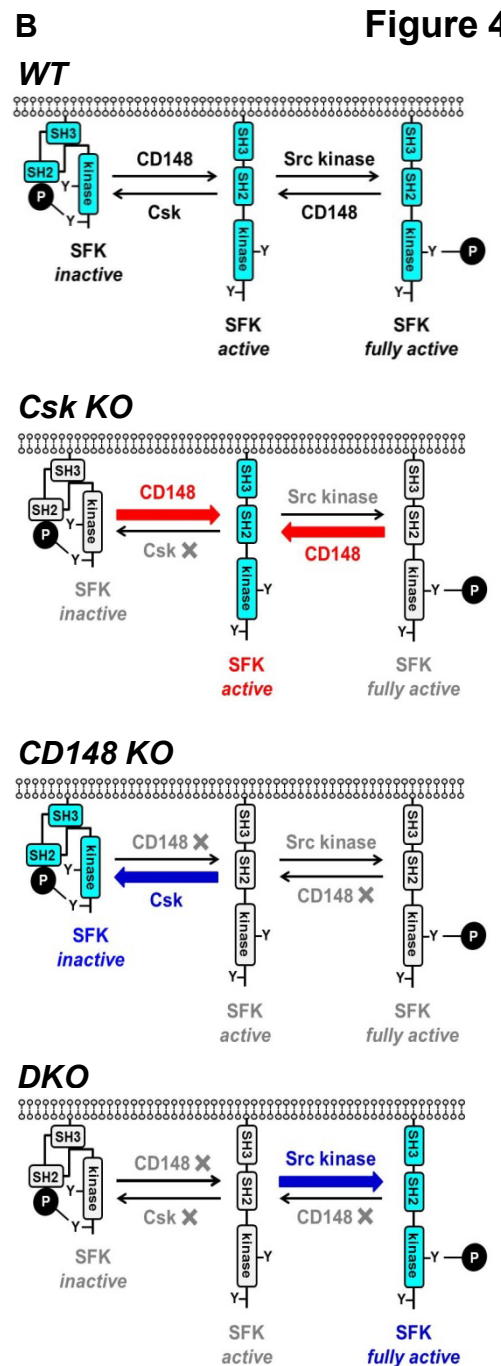
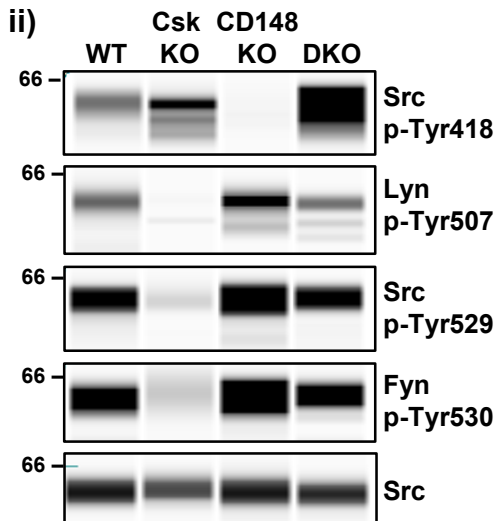
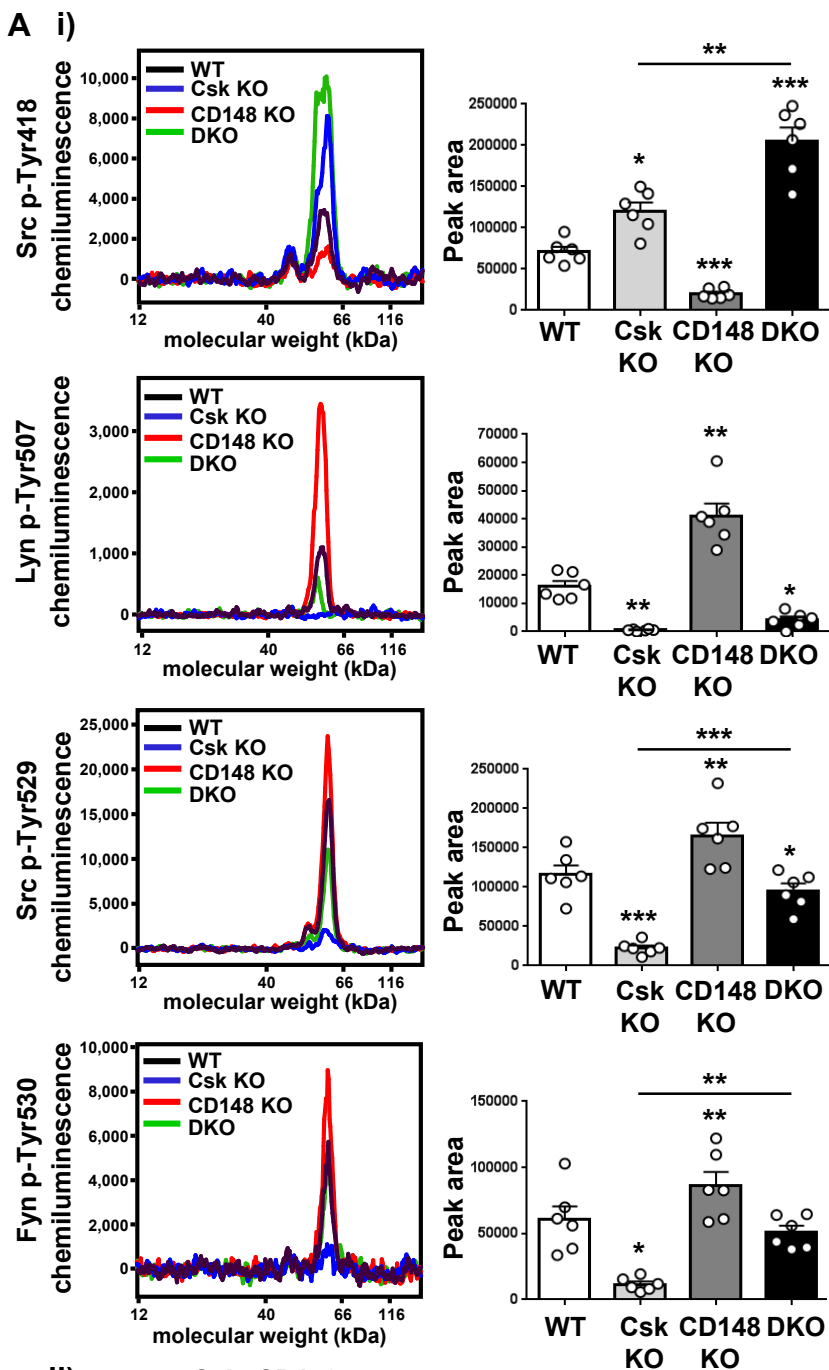


Figure 4



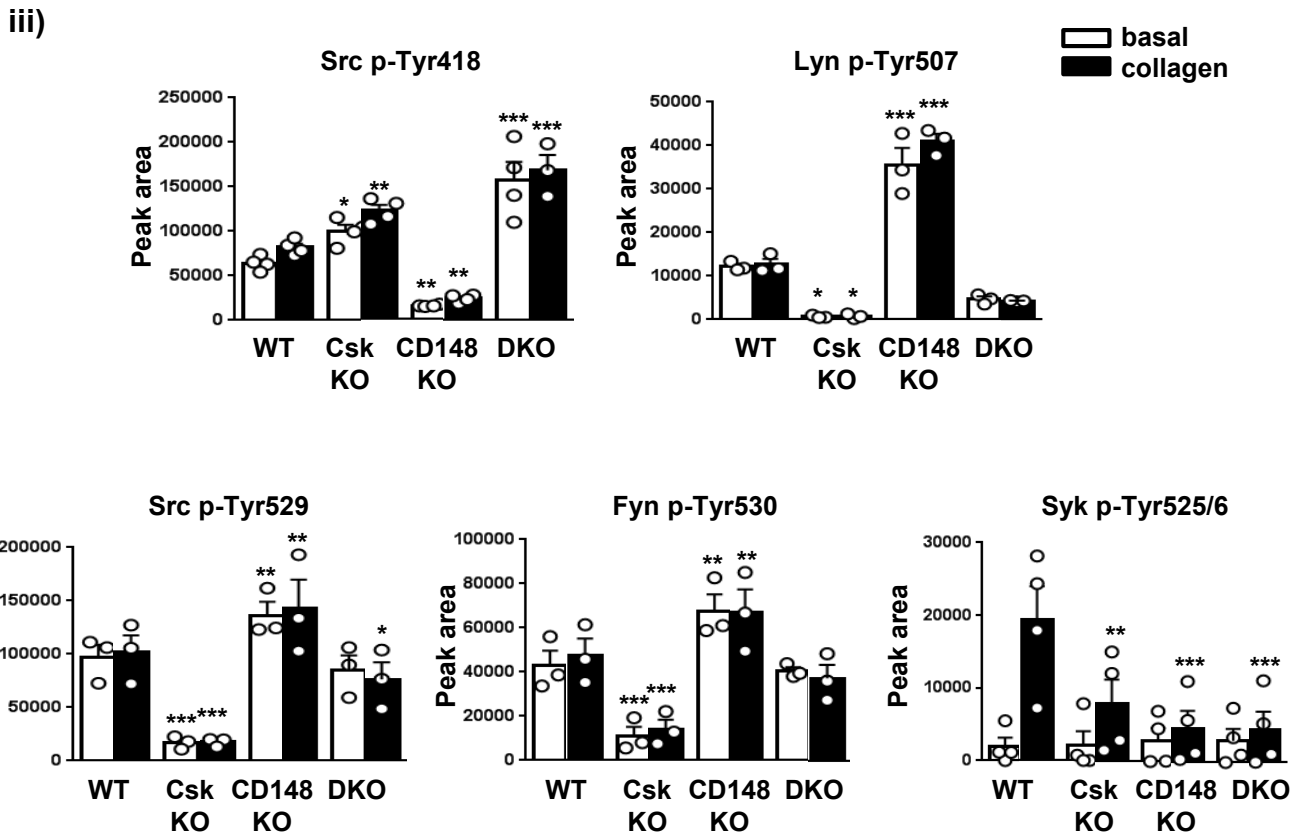
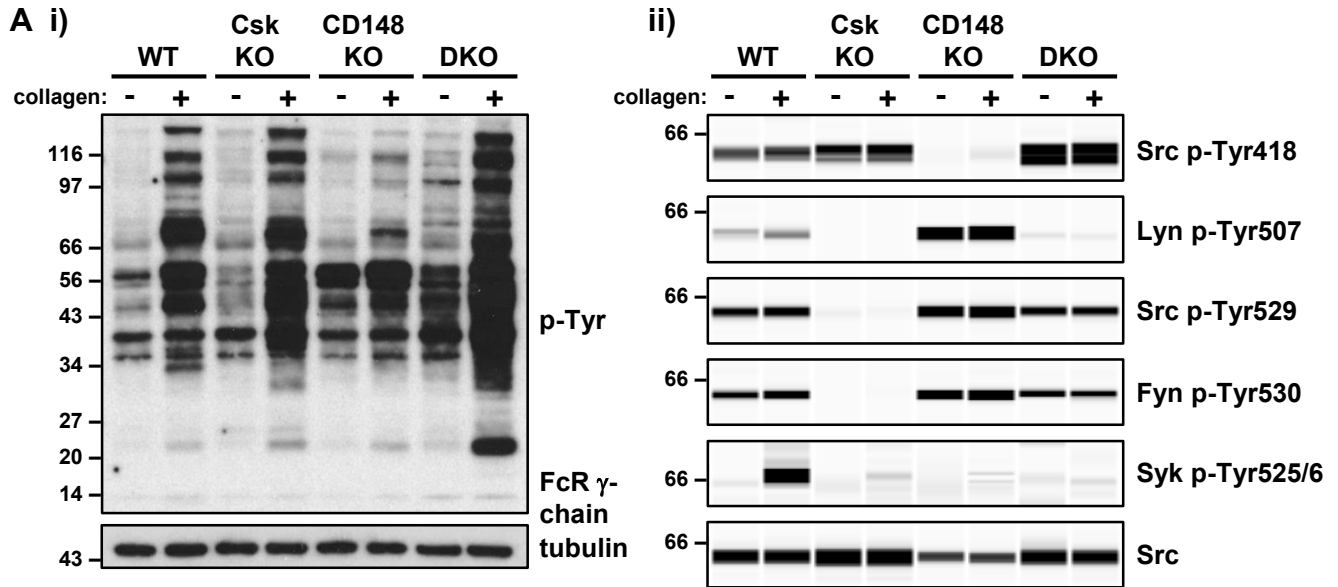
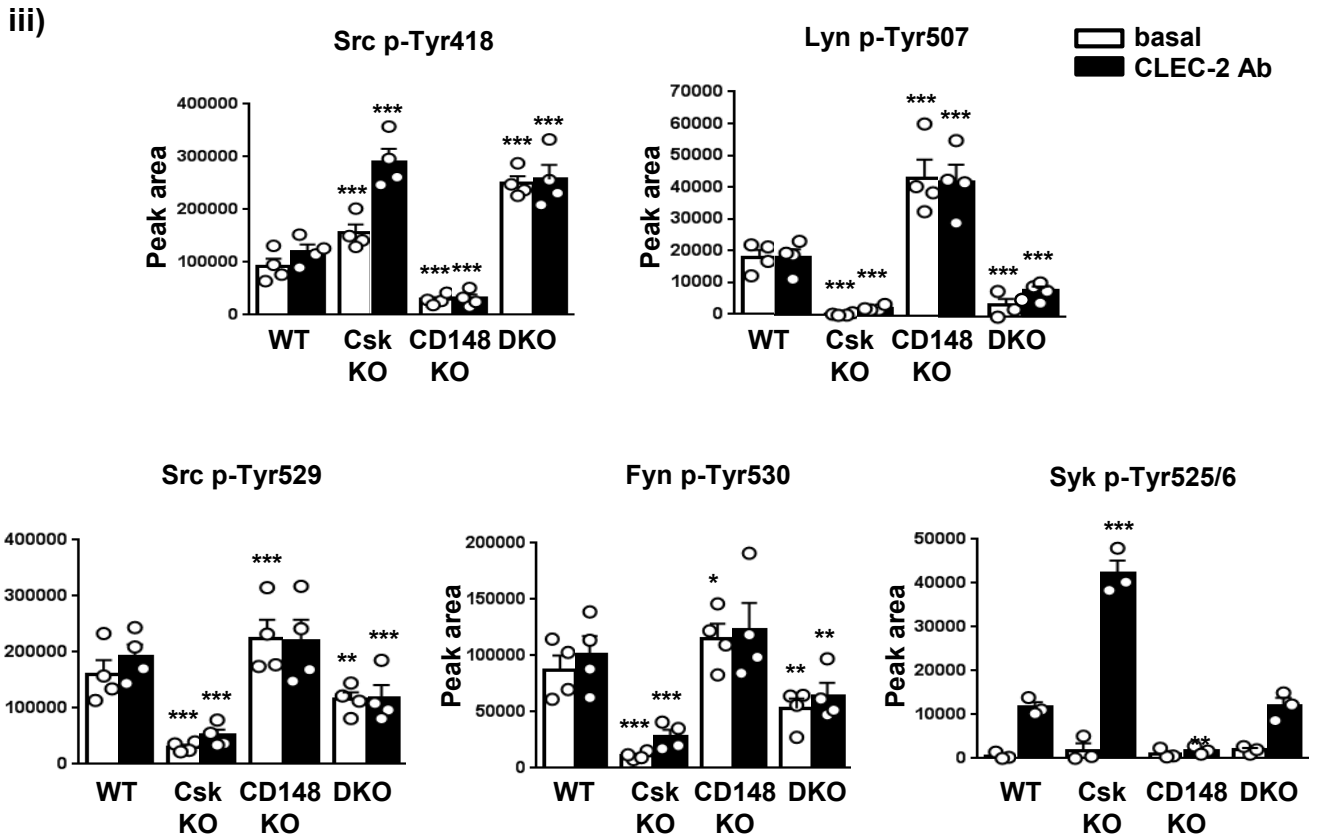
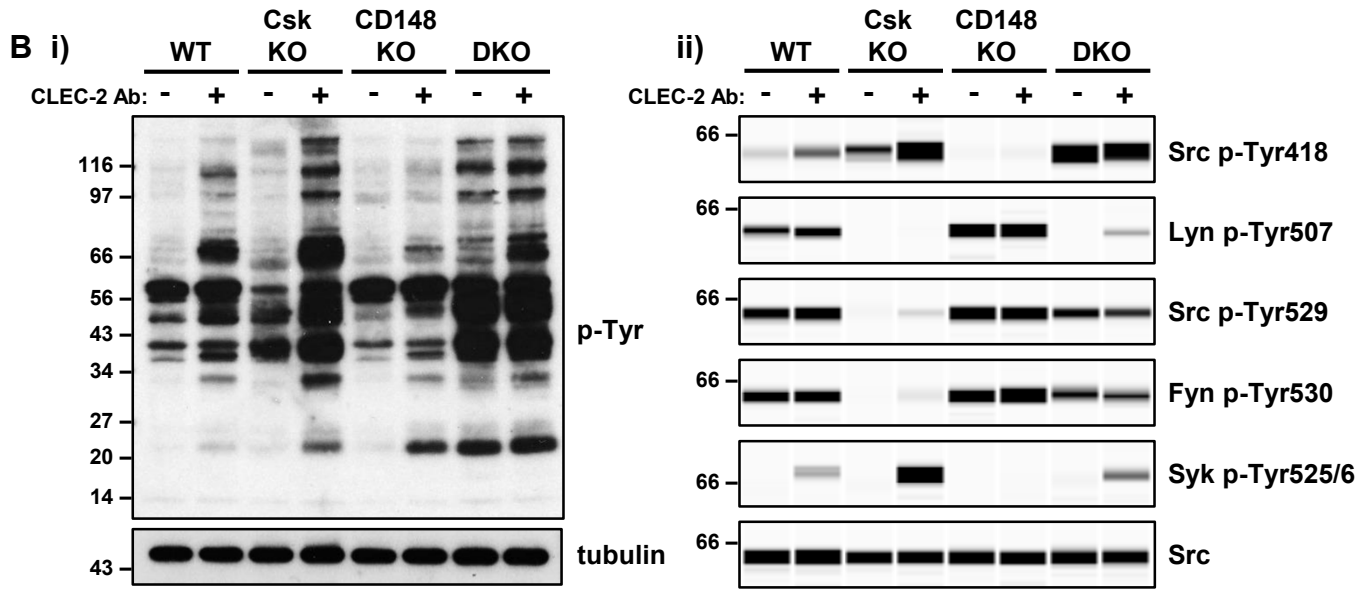
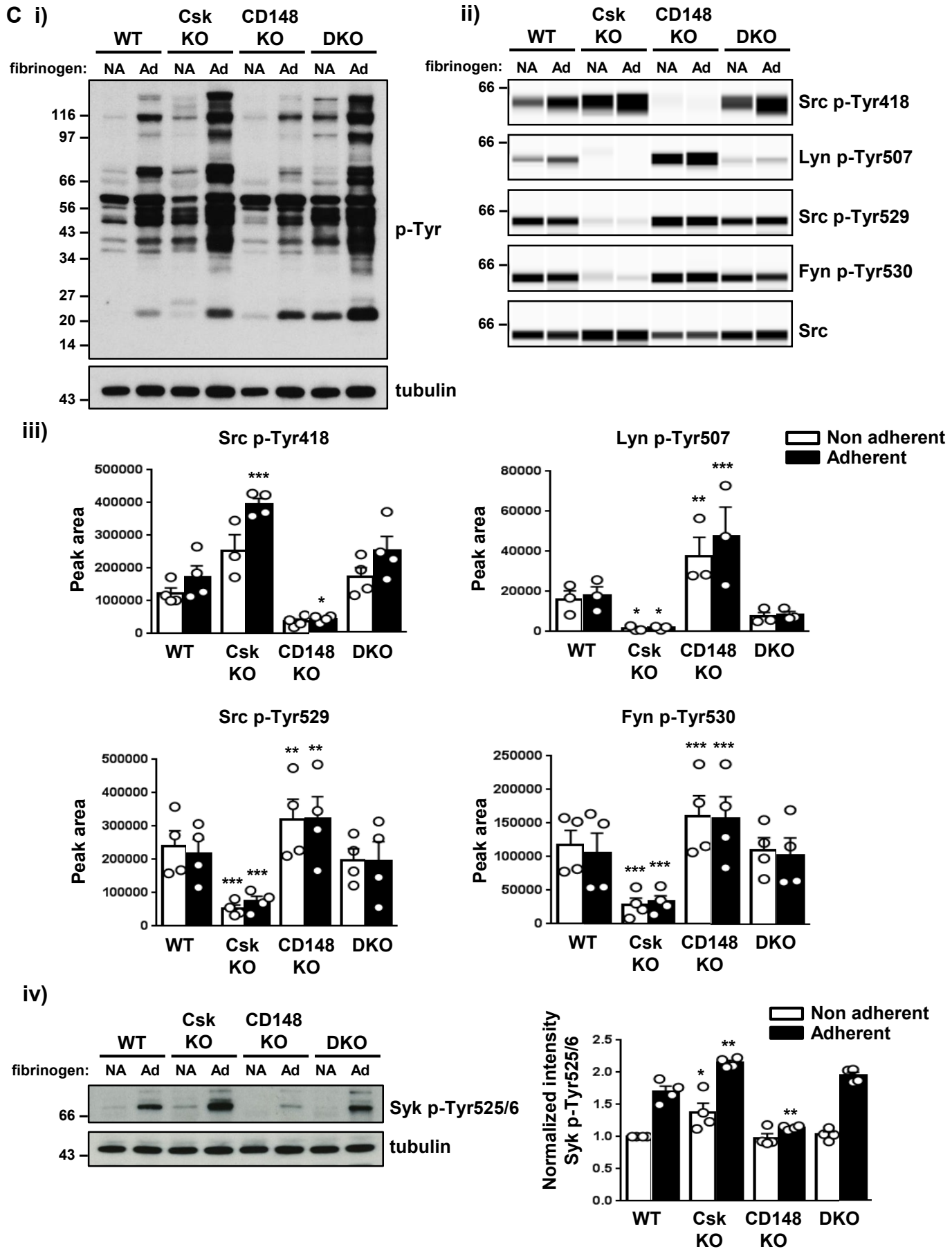


Figure 5





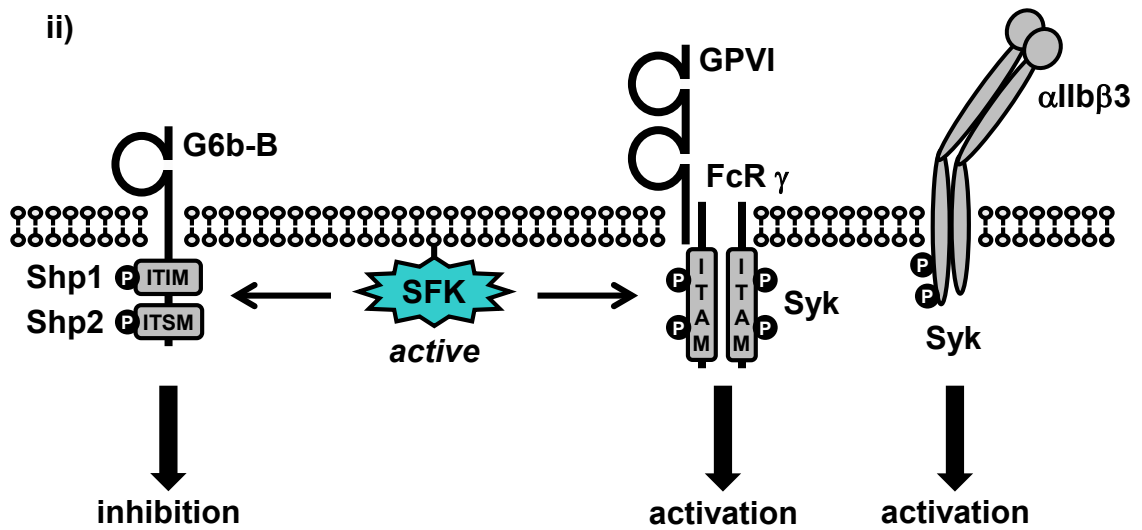
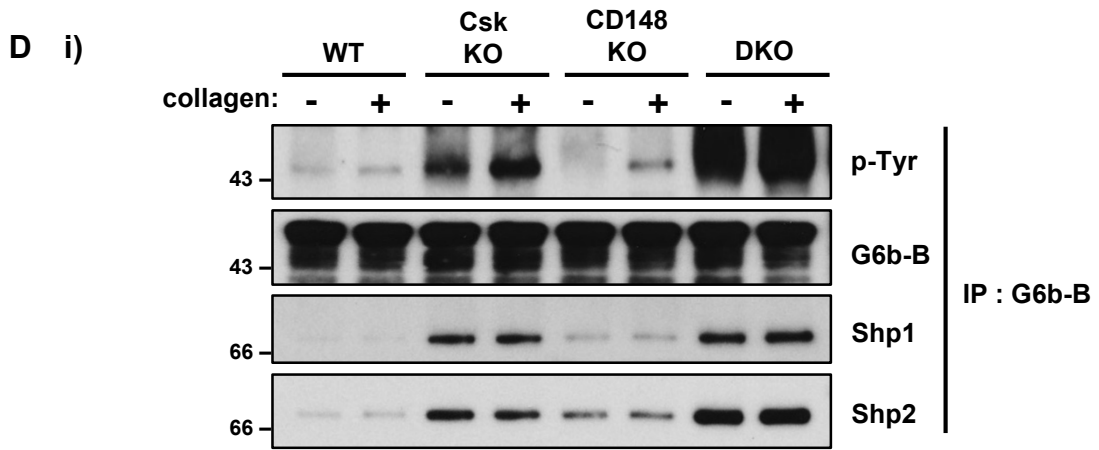


Figure 6

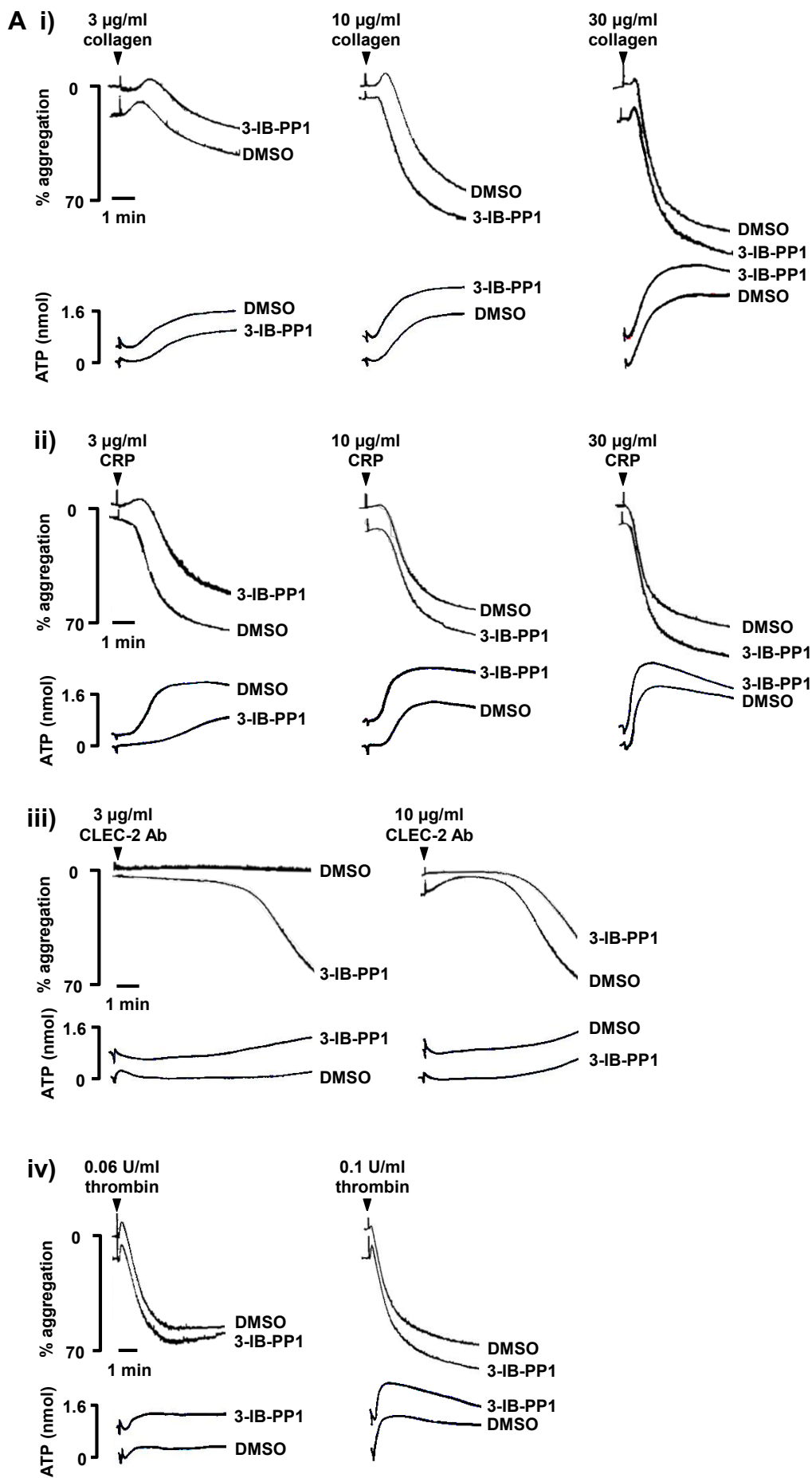
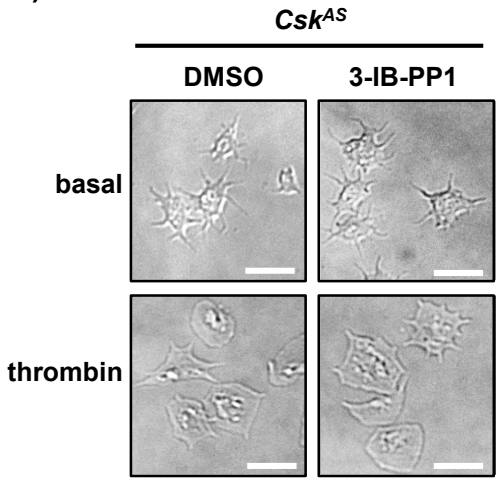


Figure 6

B i)



ii)

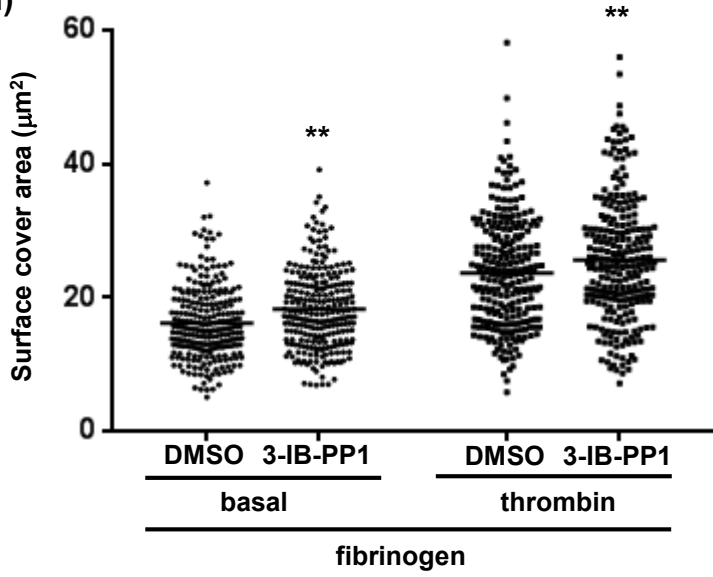
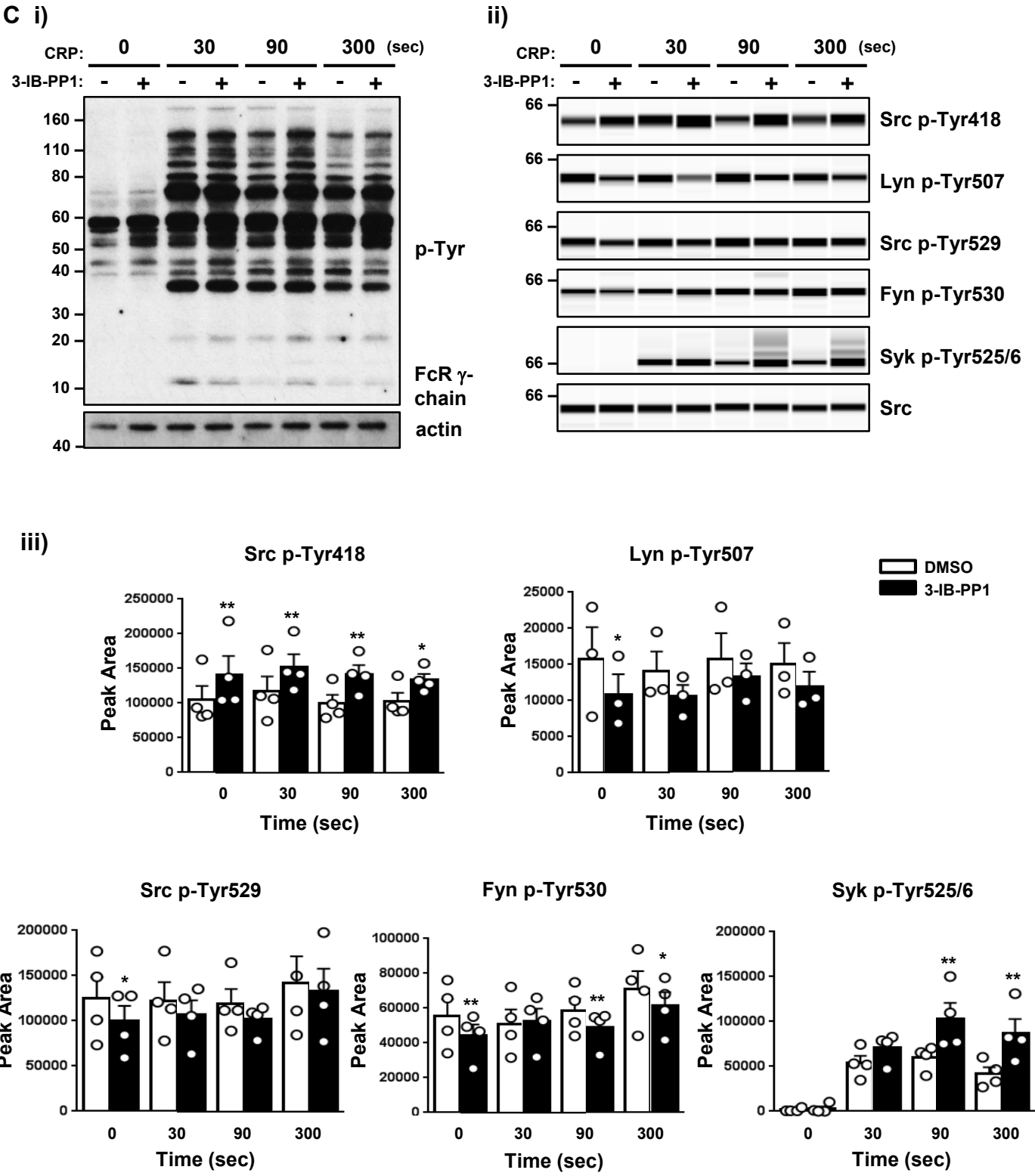
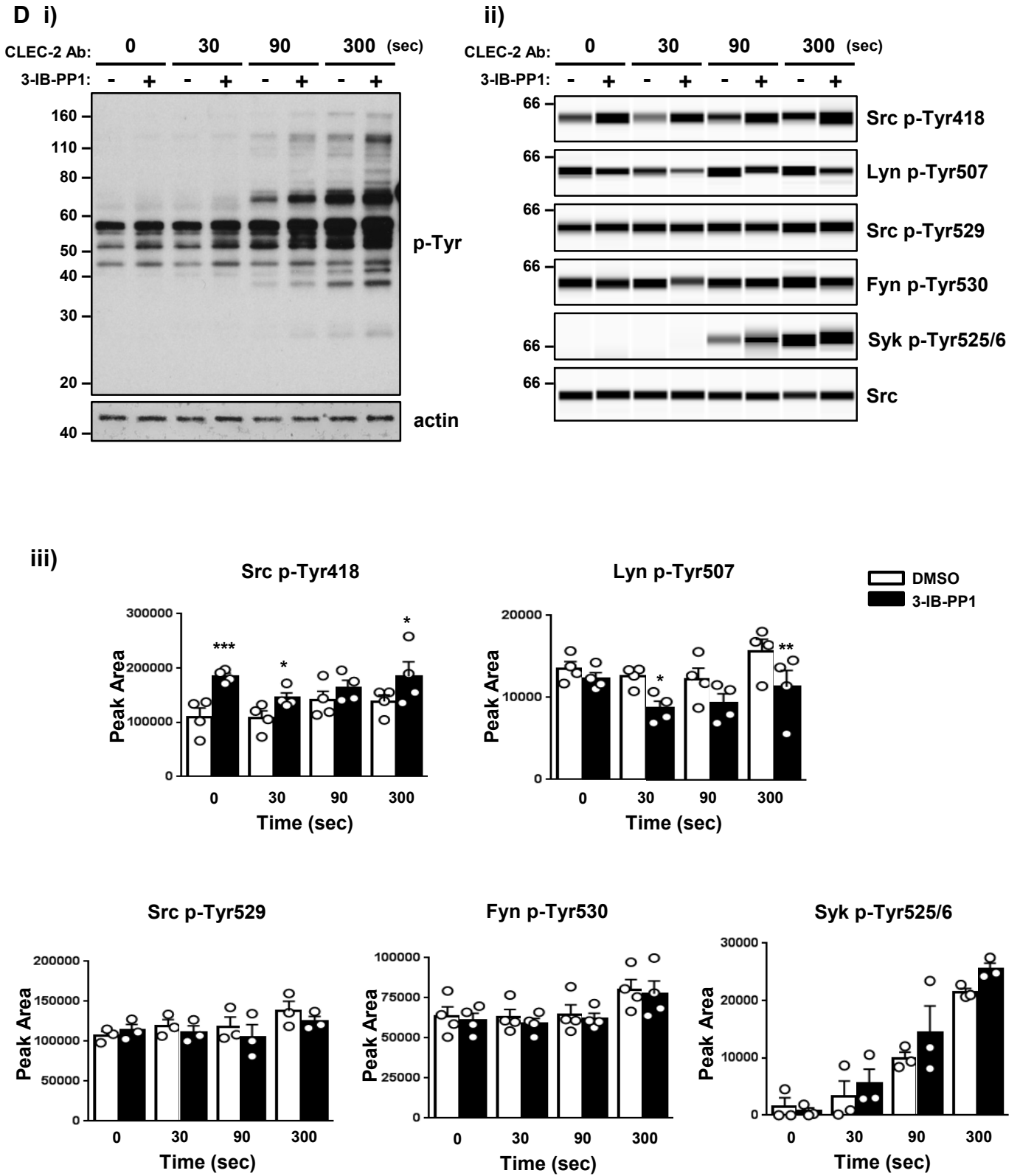


Figure 6





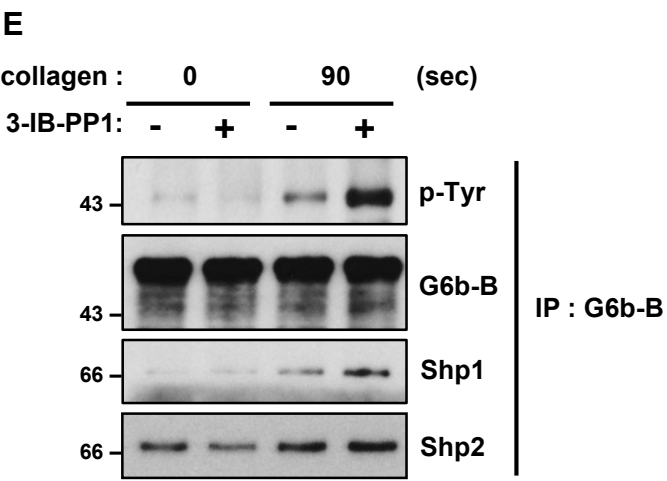
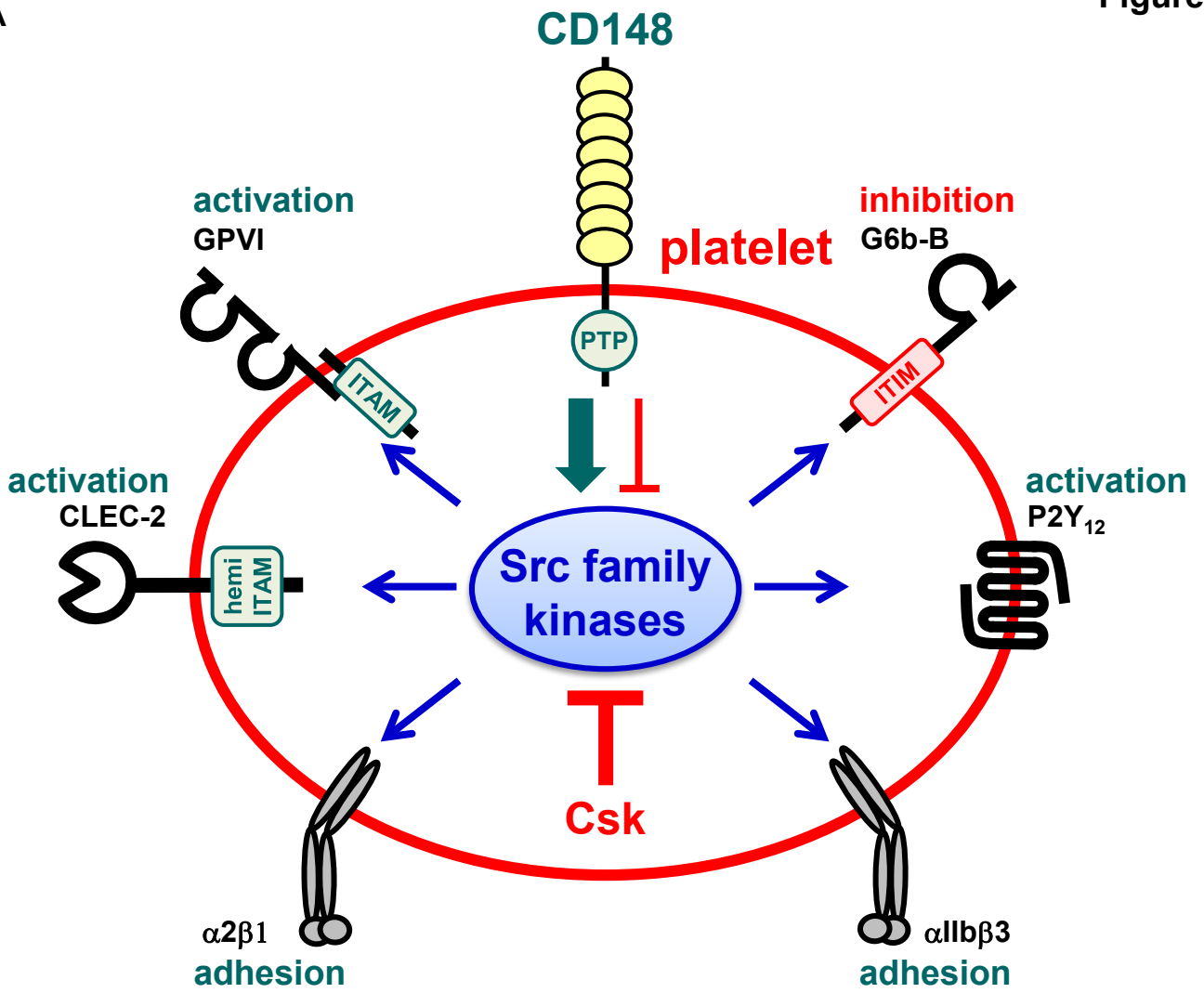
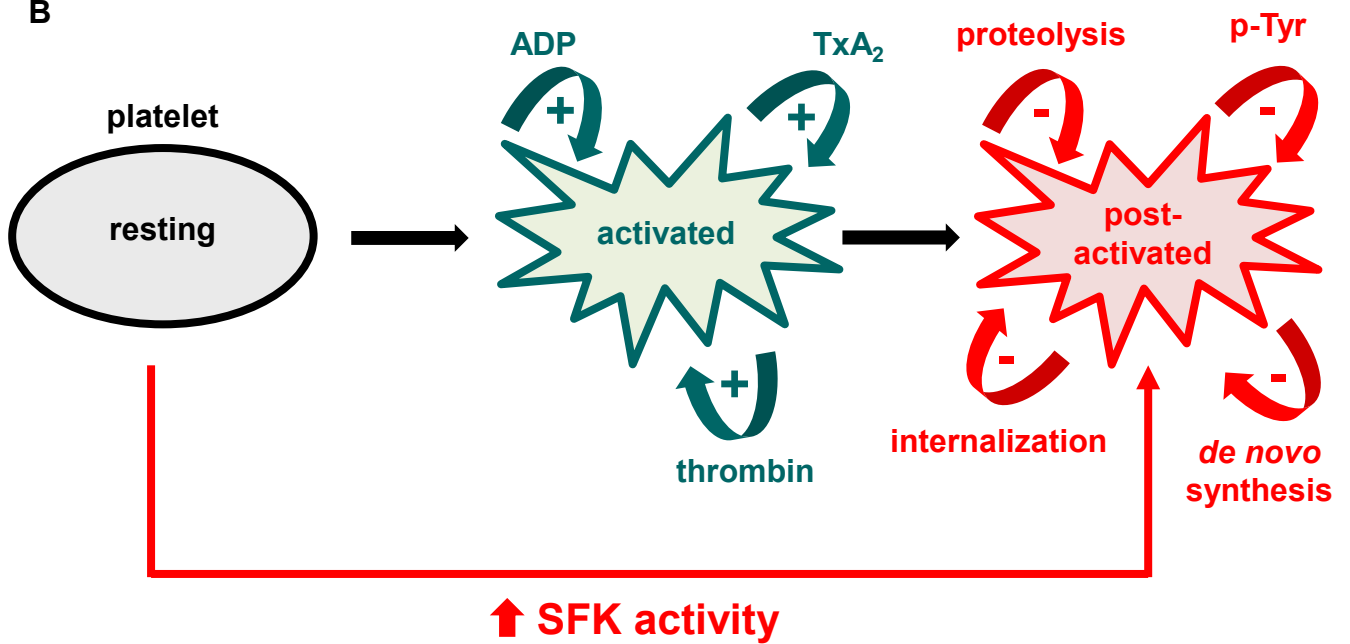


Figure 7

A



B





blood[®]

Prepublished online January 4, 2018;
doi:10.1182/blood-2017-02-768077

Maintenance of murine platelet homeostasis by the kinase Csk and the phosphatase CD148

Jun Mori, Zoltan Nagy, Giada Di Nunzio, Christopher W. Smith, Mitchell J. Geer, Rashid Al Ghaithi, Johanna P. van Geffen, Silke Heising, Luke Boothman, Bibian M. E. Tullemans, Joao N. Correia, Louise Tee, Marijke J. E. Kuijpers, Paul Harrison, Johan W. M. Heemskerk, Gavin E. Jarvis, Alexander Tarakhovsky, Arthur Weiss, Alexandra Mazharian and Yotis A. Senis

Information about reproducing this article in parts or in its entirety may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#repub_requests

Information about ordering reprints may be found online at:
<http://www.bloodjournal.org/site/misc/rights.xhtml#reprints>

Information about subscriptions and ASH membership may be found online at:
<http://www.bloodjournal.org/site/subscriptions/index.xhtml>

Advance online articles have been peer reviewed and accepted for publication but have not yet appeared in the paper journal (edited, typeset versions may be posted when available prior to final publication). Advance online articles are citable and establish publication priority; they are indexed by PubMed from initial publication. Citations to Advance online articles must include digital object identifier (DOIs) and date of initial publication.